Ecologie des chytrides parasites de la cyanobactérie Anabaena macrospora

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Écologie des chytrides parasites de la cyanobactérie *Anabaena macrospora*

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Titre : Écologie des chytrides parasites de la cyanobactérie Anabaena macrospora

Résumé

En raison des forçages anthropiques exercés sur les écosystèmes aquatiques et des effets des changements globaux présents et à venir, on s'attend à une augmentation de la fréquence et de l'intensité des proliférations cyanobactériennes lacustres. Une meilleure connaissance des facteurs biotiques, et notamment du parasitisme, impliqués dans le control des dynamiques cyanobactériennes semble essentielle. Il est désormais établi que les Chytridiomycota (chytrides) constituent les principaux pathogènes fongiques du phytoplancton. Ainsi, les travaux de recherche menés au cours de cette thèse ont eu pour objectif d'étudier le parasitisme fongique associé aux efflorescences cyanobactériennes, et plus précisément l'écologie des chytrides parasites de la cyanobactérie *Anabaena macrospora*, espèce filamenteuse présentant des proliférations massives et récurrentes dans le lac d'Aydat (France). Par la mise en place d'un schéma d'échantillonnage temporel et spatial à haute fréquence et prenant en compte deux années consécutives (2010 et 2011), nous avons pu (i) étudier les cycles de vie des deux espèces de parasites fongiques associées à *A. macrospora*, (ii) évaluer l'impact de ces parasites sur la dynamique de la population cyanobactérienne, et (iii) caractériser des facteurs contrôlant la dynamique des systèmes hôtes-parasites d'intérêt. De plus, (iv) afin de mettre en exergue les relations étroites existantes entre l'hôte et la production fongique associée, nous avons mis au point des protocoles méthodologiques permettant une analyse microscopique fine de l'hôte, des sporanges et de leur contenu en zoospores (fécondité des chytrides). Les résultats acquis mettent en évidence la coexistence de deux espèces de chytrides, *Rhizosiphon crassum* et *R. akinetum*, associées à *A. macrospora*. La reconstruction des cycles de vie par analyse d'images prises à partir d'échantillons naturels nous a permis de montrer que les deux chytrides présentaient une durée de leur cycle de vie similaire, et estimée à environ 3j. pour *R. crassum*. Cependant, ces deux espèces se différencient d'un point de vue fonctionnel en parasitant des types cellulaires distincts : *R. crassum* influence directement la biomasse active en parasitant les cellules végétatives, alors que *R. akinetum* parasite les cellules de résistances (akinètes) de *A. macrospora*. Cette dernière espèce peut donc avoir un impact sur le recrutement, la structure génétique et la variabilité interannuelle de la population hôte. Par ailleurs, outre leur impact direct, nous montrons que l'action des chytrides parasites peut aboutir à une fragmentation mécanique des filaments de *A. macrospora,* augmentant potentiellement la perte de biomasse cyanobactérienne par broutage. De plus, nous avons pu mettre en évidence que la production zoosporique des chytrides dépendait des ressources nutritives disponibles tant d'un point vue quantitatif (taille de l'hôte) que qualitatif (groupe phytoplanctonique parasité, métabolisme de l'hôte…). La réduction de la biomasse active de cyanobactéries, la fragmentation mécanique, ainsi que la production de zoospores dont la qualité nutritive pour le zooplancton a été démontrée, établissent les chytrides comme lien trophique entre les proliférations cyanobactériennes filamenteuses « *inedible* », considérée comme impasses trophiques, et les niveaux trophiques supérieurs. Enfin, l'ensemble des résultats acquis montre l'intérêt de prendre en compte, désormais, le rôle fonctionnel des champignons microscopiques parasites, notamment comme agents régulateurs direct et indirect du développement phytoplanctonique. Cette prise en compte permettrait, sans aucun doute, d'améliorer les modèles de transferts de matière et d'énergie qui transitent dans les écosystèmes aquatiques, dans le contexte général d'optimiser la gestion des plans d'eau.

Mots clefs: *Anabaena macrospora, Rhizosiphon crassum, R. akinetum, chytrids, cyanobacteria, ecology*

Titre : Ecology of chytrids parasitizing the cyanobacterium *Anabaena macrospora*

Abstract

Face to both the important anthropogenic input in nutrients and the global change, numerous authors predict that the cyanobacterial blooms will increase in relative abundance in aquatic ecosystems. An exhaustive knowledge of the driving biotic factors of the cyanobacterial dynamic is essential. In lakes, the most common fungal parasites of phytoplankton belong to the phylum Chytridyomycota (i.e. chytrids). The aim of the thesis was to investigate the fungal parasitism associated to the cyanobacterial blooms, particularly the ecology of chytrids parasitizing the filamentous cyanobacterial species *Anabaena macrospora*, in Lake Aydat (France). During two successive years (2010-2011), investigations on (i) the chytrid cycle of life of two chytrid species parasitizing *A. macrospora*, (ii) the impact of the fungal parasitism on the cyanobacterial bloom dynamic and (iii) driving factors of the host-parasite pairings dynamics have been led during two spatio-temporal surveys using high resolution sampling strategies. Moreover (iv) a double staining method based on a combination of CFW and SYTOX green for counting, identifying, and investigating the fecundity of phytoplankton fungal parasites and the putative relationships established between hosts and their fungal parasites has been developed. Results underlined the coexistence of two chytrids, *Rhizosiphon crassum* and *R. akinetum*, which have similar life cycles but differed in their infective regimes depending on the cellular niches offered by their host. *R. crassum* infected both vegetative cells and akinetes while *R. akinetum* infected only akinetes. A reconstruction of the developmental stages suggested that the life cycle of *R.crassum* was completed in about 3 days. By infecting akinetes, *R. akinetum* could reduce or modify the genetic structure of the cyanobacterial bloom of the following year. Furthermore, chytrids may reduce the length of filaments of *Anabaena macrospora* significantly by ‘‘mechanistic fragmentation’’ following infection. All these results suggest that chytrid parasitism is one of the driving factors involved in the decline of cyanobacterial blooms, by direct mortality of parasitized cells and indirectly by the mechanistic fragmentation, which could weaken the resistance of *A. macrospora* to grazing. Moreover, we underlined that the production of zoospore depends on the nutritional host quantity (host size) and quality (host phytoplanktonic group, host metabolism…). The decrease of the cyanobacterial active biomass, mechanistic fragmentation, and production of zoospores which exhibit a high nutritional quality for the zooplankton, established the chytrids as a real link between the inedible filamentous cyanobacteria, considered as trophic dead ends, and the higher trophic levels. Overall, we consider that the acquisition of our data places the chytrid parasitism as an important driving factor of the phytoplankton dynamic, allowing the inclusion of fungi and their main function (parasitism) in the energy and matter fluxes in the pelagic ecosystems.

Keywords: *Anabaena macrospora, Rhizosiphon crassum, R. akinetum, chytrids, cyanobacteria, ecology*

« Viaur gardait une conscience aussi lucide, aussi exigeante, aussi éplucheuse du détail que lorsqu'il travaillait au microscope par un de ses meilleurs jours »

-Les hommes de bonne volonté-

Jules Romains (1885-1972)

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Liste des abréviations

ADN	Acide DésoxiriboNucléique
ANOVA	ANalyse Of VAriance
ANR	Agence Nationale de la Recherche
CARD-FISH	Catalyzed Reporter Deposition Fluorescent in situ Hybridization
CF	Conversion Factor
CFW	CalcofluorWhite
DAPI	4',6-DiAmidino-2-PhénylIndole
DOM	Dissolved Organic Matter
HF	Heterotrophic Flagellate
HNF	Heterotrophic Nano Flagellate
I AK	Intensité d'infection des Akinètes
I.A.	Immature Akinete
I.C.N.B.	International Code of Botanical Nomenclature
I.C.P.N	International Code of Nomenclature of Prokaryotes
M.A	Mature Akinete
MF	Maximum of Fluorescence
PCC	Pasteur Culture Collection of Cyanobacteria
PrAK	Prevalence of infection of akinete
PrC	Prevalence of infection of cells
PrCF	Prevalence of infection of cells in infected filament
PrF	Prevalence of infection of filament
PSI	Photosystem I
PSII	Photosystem II
qPCR	Quantitative Polymerase Chain Reaction
R.A	Reproduction asexuée
R.S	Reproduction sexuée
UV	Ultra-violet

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Figure 6 : *Rhizosiphon crassum* parasitant un akinète (flèche noire) et développant un système rhizoïdal tubulaire (flèche blanche) au travers de plusieurs cellules d'un filament cyanobactérien d'*Anabaena macrospora*.

Figure 7 : Photographies issues d'observation au MET d'un akinète infecté par des virus à queue (siphoviridae) (flèche noire pleine)(A, B) et de deux cellules végétatives parasitées par le système rhizoïdal tubulaire de *Rhizosiphon crassum* (flèche noire vide)(C, D). Echelle : 100nm (A, B) ; 1µm (C,D). Photo : Jonathan Colombet.

Tableau 1 : Comparaison relative des deux espèces fongiques parasites de la cyanobactérie *Anabaena macrospora* dans un modèle r et K. *Rhizosiphon crassum* est représenté par la couleur beige, *R. akinetum* par la couleur violette. Lorsqu'aucune couleur ne figure, ceci signifie que les données ne sont pas connues. (Les caractéristiques des deux stratégies sont issues d'une étude menée par Barbosa, 1977).

Introduction générale

La compréhension du fonctionnement des réseaux trophiques aquatiques passe inéluctablement par la connaissance de l'ensemble des communautés qui les compose. Comme le souligne Pomeroy *et al.,* (2007) deux siècles ont été nécessaires depuis Darwin et ses questionnements sur la vie microbienne, pour reconnaitre toute l'importance et la diversité des microorganismes dans les réseaux trophiques aquatiques. Ainsi, alors que jusqu'ici la linéarité des flux de matière était acceptée, en 1983 le concept de boucle microbienne proposé par Azam (1983) a modifié la vision classique de la chaine trophique : phytoplancton → zooplancton → poisson. Cela a amené à reconsidérer l'importance des communautés bactériennes, ciliées et de flagellés hétérotrophes (HNF) dans les réseaux trophiques aquatiques. Au sein de ce concept, la composante flagellée (HNF), principalement considérée comme bactérivore, joue un rôle primordial en transférant la matière assimilée (matière organique dissoute et particulaire provenant du phytoplancton et du zooplancton) par les procaryotes hétérotrophes, aux niveaux trophiques supérieurs. Cependant, au sein de ces flagellés hétérotrophes, de nombreuses espèces restaient non identifiées et ne semblaient pas être capables de « bactérivorie » (Wieltschnig*, et al.*, 2001). L'évolution de l'écologie microbienne *via* notamment l'avancement des méthodes moléculaires a permis d'identifier ces flagellés méconnus et a ainsi mis en exergue l'importance de formes libres de champignons et d'alvéolés, les zoospores, dans la composante des flagellés hétérotrophes (Lefèvre*, et al.*, 2006, Lefèvre*, et al.*, 2008, Lepère*, et al.*, 2008). Lors de ces diverses études, les principaux champignons zoosporiques retrouvés ont été phylogénétiquement affiliés au phylum des Chytridiomycota et les séquences d'Alveolata, aux Perkinsozoa. Or ces deux groupes d'eucaryotes (Chytridiomycota et Perkinsozoa) ne sont pas bactérivores mais majoritairement parasites. Ainsi, les Perkinsozoa sont reconnus comme

parasites de bivalves, de poissons, et d'espèces phytoplanctoniques (Mangot*, et al.*, 2011). Les Chytridiomycota, quant à eux, sont connus dans les écosystèmes aquatiques comme principaux pathogènes fongiques du phytoplancton (Rasconi*, et al.*, 2011). Ainsi, dans les écosystèmes lacustres, la composante flagellée est loin d'être fonctionnellement homogène (Sime-Ngando*, et al.*, 2011).

Alors même qu' Hechinger *et al.,* (2011) rapportent qu'au moins la moitié de la diversité spécifique serait due aux espèces parasites, l'incorporation de ces dernières dans les modèles mathématiques caractérisant les flux de matière dans les réseaux trophiques n'est que très récente (Lafferty*, et al.*, 2008, Dunne*, et al.*, 2013). Dunne et ses collaborateurs (2013) ont ainsi pu montrer que l'ajout des parasites dans les modèles augmentait la connectance du système, particulièrement lorsque des liens concomitants étaient inclus (i.e. les liens existants entre les prédateurs et les parasites d'une même proie). Au sein des écosystèmes aquatiques, des études récentes ont modélisé, et ainsi mis en évidence, l'importance des parasites fongiques appartenant au phylum des Chytridiomycota, i.e. chytrides, dans les flux de matière des réseaux trophiques lacustres (Grami*, et al.*, 2011, Niquil*, et al.*, 2011). Ces modèles ont montré notamment que les parasites fongiques tendaient à renforcer la stabilité des réseaux trophiques. De plus, la prise en considération des chytrides dans les écosystèmes aquatiques augmente les flux de carbone et améliore le transfert de cet élément vers les niveaux trophiques supérieurs, ceci est notamment dû à l'assimilation des formes libres de ces champignons (i.e. zoospores). Les formes libres des parasites sont souvent vulnérables face aux prédateurs (Johnson*, et al.*, 2010). Dans les écosystèmes aquatiques, il a récemment été montré que les zoospores des chytrides, au même titre que les autres HNF, étaient activement broutées par le zooplancton (Kagami*, et al.*, 2004). Ces travaux ont permis de proposer en 2007 un nouveau concept écologique venant s'ajouter à la chaine trophique linéaire et au concept de boucle microbienne: la « mycoloop » (Kagami*, et al.*, 2007). Au sein de ce concept, les espèces phytoplanctoniques de grande taille, « *inedible* » par les niveaux trophiques supérieurs, sont préférentiellement parasitées par les chytrides (Ibelings*, et al.*, 2004, Kagami*, et al.*, 2007). Les zoospores issues de la phase parasitaire du cycle de vie des chytrides (i.e. les sporanges), sont quant à elles facilement ingérables par le zooplancton, et permettent ainsi de transférer la matière et l'énergie du phytoplancton de grande taille considérées jusqu'alors comme perdues pour le système, vers les niveaux trophiques supérieurs (Kagami*, et al.*, 2007). Cette boucle fongique donne aux chytrides une fonctionnalité importante, en les positionnant comme véritable lien trophique entre les producteurs primaires et le zooplancton (Sime-Ngando, 2013).

Par ailleurs, de par leur phase parasitaire, les chytrides sont connus pour être un facteur biotique important responsable du déclin de proliférations phytoplanctoniques (Sen, 1988, Van Donk & Bruning, 1992, Kagami & Urabe, 2002). Les chytrides favorisent ainsi les successions phytoplanctoniques dans les écosystèmes lacustres (Van Donk, 1989). Les nombreuses études menées sur ces parasites se sont principalement intéressées à l'effet des chytrides sur la dynamique des populations des microalgues eucaryotes (Sen, 1988, Kagami & Urabe, 2002, Rasconi*, et al.*, 2012) ; et notamment des diatomées (Van Donk & Ringelberg, 1983, Sen, 1987, Bruning, 1991, Bruning, 1991, Holfeld, 2000, De Bruin*, et al.*, 2008, Ibelings*, et al.*, 2011). En comparaison, leur rôle sur la composante phytoplanctonique procaryote, les cyanobactéries, n'a été que très peu étudié, alors même qu'il est connu depuis plus d'un siècle (Braun, 1856).

Dans les écosystèmes lacustres, les cyanobactéries peuvent constituer jusqu'à 99% de la biomasse totale du phytoplancton en période estivale. Aux vues de l'augmentation du forçage anthropique exercé sur ces écosystèmes et des changements globaux présents et à venir, ces proliférations ou « blooms » pourraient voir leur fréquence ainsi que leur intensité fortement augmentées (Elliott, 2011). Ces proliférations causent d'importants problèmes socio-économiques et écologiques. De nombreuses études se sont attachées à déterminer les facteurs favorisant ces blooms et l'apport excessif en nutriments fut clairement identifié comme un facteur abiotique primordial (Pick & Lean, 1987, Paerl, 2008, Davis*, et al.*, 2009). Dans les écosystèmes la composante biologique est également déterminante dans le contrôle des populations. Ainsi, plusieurs facteurs biotiques comme la lyse, la prédation ou encore le parasitisme sont également déterminants. Si l'impact des virus (Suttle, 2002), des bactéries lytiques (Ren*, et al.*, 2010) ou encore du zooplancton (Wilson*, et al.*, 2006) sur la dynamique cyanobactérienne est désormais établi, celui du parasitisme fongique reste encore largement méconnu. Or, comme nous l'avons discuté par ailleurs, les parasites peuvent être considérés comme un véritable lien dans le transfert de matière. Ceci donne d'autant plus d'importance à ce compartiment si l'on considère que les cyanobactéries, notamment les formes filamenteuses, de par leur taille, leur toxicité potentielle ou leur faible qualité nutritionnelle, peuvent être considérées comme une impasse trophique. Une meilleure connaissance du parasitisme fongique lié aux blooms cyanobactériens semble donc nécessaire afin d'améliorer notre compréhension du fonctionnement des réseaux trophiques mis en place au cours de proliférations cyanobactériennes.

Ainsi, au cours de cette thèse, nous nous sommes attachés, dans un premier temps, à faire une synthèse de la littérature existante sur les deux entités biologiques impliquées dans la relation parasitaire étudiée au cours de cette thèse : les cyanobactéries et les Chytridiomycota,

ainsi que sur les différents facteurs biotiques impliqués dans le déclin des proliférations cyanobactériennes (**Chapitre 1**). Grâce à la microscopie optique et au développement de techniques de coloration exposées dans le **Chapitre 2**, nous avons pu contribuer au manque de connaissances révéler lors de l'analyse bibliographique, manque de connaissance en grande partie lié aux difficultés méthodologiques. Ainsi, nous avons cherché à déterminer la dynamique spatio-temporelle de couples hôte-parasites présents dans un écosystème lacustre eutrophe, ainsi que les facteurs l'influençant, et étudié les cycles de vie des chytrides associés aux blooms de la cyanobactérie *Anabaena macrospora*, au cours de deux années successives (**Chapitre 3**). Par ailleurs, nous nous sommes attachés à étudier les relations plus étroites reliant l'hôte et le parasite notamment en nous intéressant aux paramètres influençant la fécondité des chytrides dans les écosystèmes lacustres (**Chapitre 4**). Enfin, une discussion de ces résultats ainsi que les perspectives émanant de ce travail de thèse sont présentées dans le dernier chapitre (**Chapitre 5**).

Chapitre 1

Synthèse bibliographique

1ère partie : Etat de l'art des deux microorganismes impliqués dans la relation parasitaire : les cyanobactéries et les chytrides

I. Les cyanobactéries

Les cyanobactéries constituent les plus anciennes formes de vie dont les fossiles aient pu être mis en évidence sur Terre. Ces procaryotes photosynthétiques sont apparus il y a 3.5Ga dans les milieux océaniques. Ils sont à l'origine de l'oxygénation de l'atmosphère (2.5Ga) et de fait, ont joué un rôle primordial dans l'apparition des premières formes de vie aérobie (Canfield, 2005). Par ailleurs, l'hypothèse évolutive dite de l'endosymbiose fait de ces organismes, des éléments essentiels pour l'apparition et le développement des eucaryotes photosynthétiques. En effet, l'organite clef du processus photosynthétique (le chloroplaste) est un vestige de cyanobactérie (Kutschera & Niklas, 2005). Au cours de l'évolution, les cyanobactéries se sont diversifiées et ont colonisé divers écosystèmes aux conditions physicochimiques contrastées. Ainsi, les cyanobactéries sont retrouvées, des sources d'eau chaude de Yellowstone (Papke*, et al.*, 2003) aux eaux gelées des lacs polaires (Vincent, 2002), des terres arides du plateau du Colorado (Garcia-Pichel*, et al.*, 2001) aux sols humides de l'arctique (Comte*, et al.*, 2007).

1) Organisation structurelle des cyanobactéries

Ces microorganismes sont des bactéries Gram négatif (une membrane interne, une externe et une paroi fine pauvre en peptidoglycane) et arborent les caractéristiques propres aux procaryotes tels que l'absence de noyaux et d'organite comme les mitochondries ou les plastes. Cependant, tout comme les eucaryotes photosynthétiques, les cyanobactéries convertissent l'énergie lumineuse en énergie chimique *via* le processus de la photosynthèse effectué par un photosystème complexe, composé de deux photosystèmes (PSI et PSII), localisé dans la membrane des thylakoïdes, et ont la capacité de synthétiser de la chlorophylle *a* et des carotenoïdes. *A contrario* des eucaryotes, chez les cyanobactéries, la majeure partie de la captation de la lumière s'effectue à l'extérieur de la membrane des thylakoïdes par des antennes photocollectrices, appelées phycobilisomes *via* des pigments accessoires nommés phycobiliprotéines, regroupant la phycocyanine, la phycoérythrine, l'allophycocyanine et la phycoerthrocyanine.

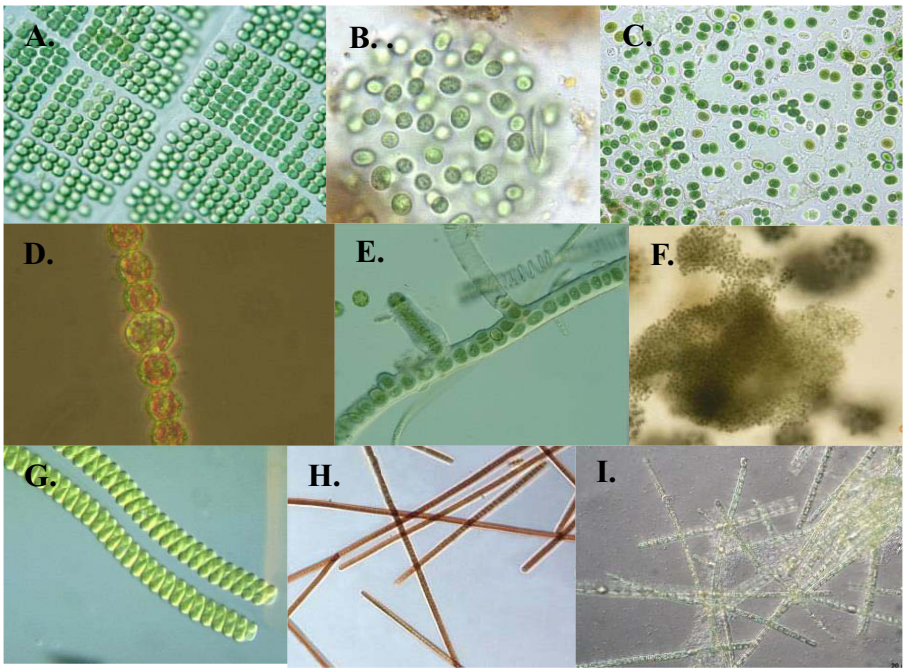

Figure 1: Illustration de la diversité morphologique des cyanobactéries. A. *Merismopedia glauca* B. *Aphanothece sp.* C. *Gloethece sp.* D. *Anabaena macrospora* E. *Stigonema minutum* F. *Microcystis ichtyoblabe* G. *Spirulina sp*. H. *Planktotriks sp.* I. *Aphanizomenon klebahnii*

Tableau 1:

Classification des cyanobactéries selon le code international de nomenclature procaryote (I.C.P.N) et botanique (I.C.B.N.)

Classification du I.C.P.N.	Classification du I.C.B.N.	Morphologie	Mode de reproduction	Cellules différenciées
Section I	Chroococcales	Unicellulaire ou en agrégats maintenus par une matrice gélatineuse	Division binaire ou bourgeonnement	Formation rare d'akinètes
Section II	Pleurocapsales		Fissions multiples internes avec production de cellules plus petites, ou ensemble de fissions multiples et binaires	
Section III	Oscillatoriales	Filamenteuse unisériée sans ramification	Division binaire dans un seul plan	Absence d'hétérocyste et d'akinète
Section IV	Nostocales			Formation d'hétérocystes
		Trichome	Division binaire	

2) Une nomenclature en pleine évolution

Les cyanobactéries se regroupent en quelques 2000 espèces réparties en 150 genres (Duy*, et al.*, 2000). En outre, elles présentent une diversité morphologique considérable (Fig. 1) sur laquelle repose, en partie, leur classification dans le code international de nomenclature de botanique (I.C.B.N) (Greuter*, et al.*, 2000). Ce dernier différencie cinq grands ordres : Les Chroococcales, les Pleurocapsales, les Oscillatoriales, les Nostocales, et les Stigonematales. (Tableau 1). Durant des décennies, les cyanobactéries étaient nommées algues bleues ou cyanophycées ce qui explique leur prise en compte dans l'I.C.B.N. Cependant, reconnaissant leur appartenance au domaine des *Bacteria*, Stanier *et al.,* proposèrent en 1978 que leur nomenclature soit régie par le code international de nomenclature des procaryotes (I.C.P.N) (Stanier*, et al.*, 1978). L'I.C.P.N propose une nomenclature différente basée non pas sur la morphologie et le mode de reproduction comme dans l'I.C.B.N, mais sur des caractères physiologiques et génotypiques de culture pure (voir Tableau 1). Bien que leur nomination diffère, les deux codes reconnaissent les mêmes subdivisions, les divergences n'apparaissant qu'à des niveaux taxonomiques inférieurs (genre et/ou espèce). La prise en compte des cyanobactéries par ces deux codes amène à de nombreux synonymes pour la même entité taxonomique. Récemment, la nécessité de convergence des deux nomenclatures a été soulevée par Hoffmann *et al.*, (2005) et explicite l'importance de considérer l'interdisciplinarité (génétique, ultrastructure, physiologie, et écologie) pour la mise en place d'une nouvelle classification cyanobactérienne.

3) Les cyanobactéries : un ensemble de cellules végétatives et différenciées

Au sein des cyanobactéries, les groupes des Nostocales et des Stigonematales peuvent produire des cellules spécialisées nommées hétérocystes et akinètes dont les rôles respectifs sont de faire face à la limitation en azote et aux conditions environnementales défavorables.

a) Les hétérocystes

Les hétérocystes sont des cellules différenciées dont le rôle est de subvenir aux besoins en azote de la colonie lorsque le milieu est carencé en ce nutriment. Pour ce faire, les hétérocystes possèdent de la nitrogénase leur permettant de fixer l'azote atmosphérique (N_2). L'activité de cette enzyme nécessite un environnement très faiblement oxygéné. De ce fait, la création d'un environnement microaérobie est indispensable et impose de nombreuses

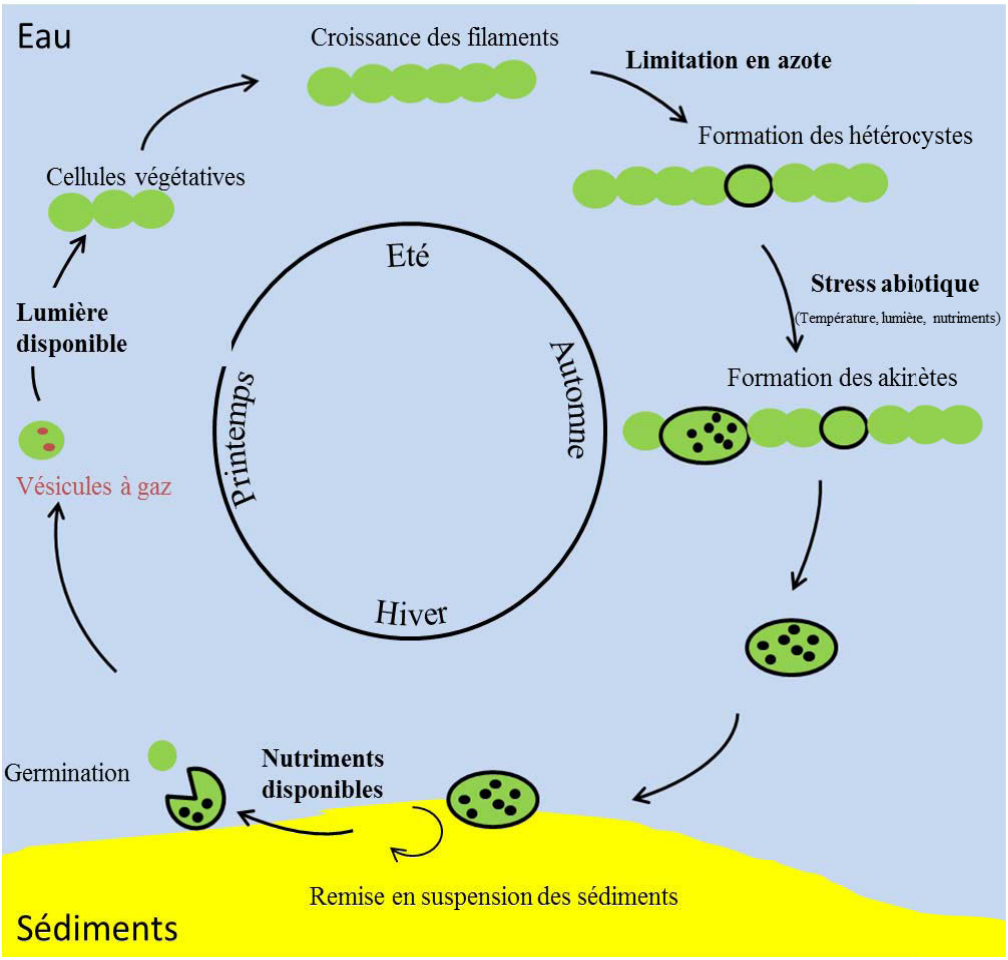

Figure 2 : Schéma du cycle de vie des cyanobactéries appartenant à l'ordre des Nostocales. D'après Hense and Beckmann (2006)

modifications structurelles et métaboliques lors de la différenciation des cellules végétatives en hétérocystes. Tout d'abord, leur paroi s'épaissit afin de diminuer la diffusion des gaz. De plus, la synthèse d'oxygène par le processus de photosynthèse est rendue impossible en raison de la perte d'activité du PSII. Enfin, les traces d'oxygène restantes sont éliminées grâce à de nombreux autres mécanismes, dont le plus important est certainement la respiration (Adams & Duggan, 1999).

Les hétérocystes sont produits lorsque le milieu devient carencé en azote. Ainsi, les cyanobactéries capables de produire ce type de cellules acquièrent un avantage compétitif important par rapport aux autres cellules phytoplanctoniques. Du fait de leur très haut degré de spécialisation, les hétérocystes sont tributaires du métabolisme des cellules végétatives adjacentes pour leur alimentation énergétique (Wolk*, et al.*, 2004). Ceci expliquerait le fait qu'aucune cyanobactérie unicellulaire ne soit capable de différencier des hétérocystes.

b) Les akinètes

Véritables spores pouvant s'apparenter aux endospores d'autres procaryotes ou eucaryotes (Kaplan-Levy*, et al.*, 2010), les akinètes assurent la pérennité des espèces cyanobactériennes filamenteuses hétérocystées et constituent une forme de dissémination importante pour la colonisation des milieux. Ces spores de résistance sont les seules cellules ayant la capacité de résister aux conditions climatiques défavorables. Elles permettent la recolonisation de la colonne d'eau lorsque les conditions deviennent de nouveau propices au développement des cyanobactéries (Fig.2).

La différenciation d'une cellule végétative en akinète est conditionnée par de nombreux facteurs abiotiques tels que la lumière, la température et les nutriments. Sutherland (1979) et Nichols & Adams (1982) ont ainsi pu mettre en évidence que de faibles intensités lumineuses induisaient la différentiation des akinètes chez les genres *Anabaena* et *Nostoc*. *A contrario* la formation d'akinètes chez le genre *Cylindrospermopsis* semble être inférée à des intensités lumineuses élevées (Moore*, et al.*, 2005). De plus, la qualité de la lumière jouerait également un rôle important dans la différenciation de ces spores. Cependant, là encore, les conclusions diffèrent, les longueurs d'onde favorisant la différenciation semblent être liées au genre considéré puisque la lumière verte stimule ce mécanisme chez l'espèce *Gleotrichia echinulata* alors que la lumière rouge activerait la différenciation des akinètes d'*Anabanea circinalis* (Thompson*, et al.*, 2009).

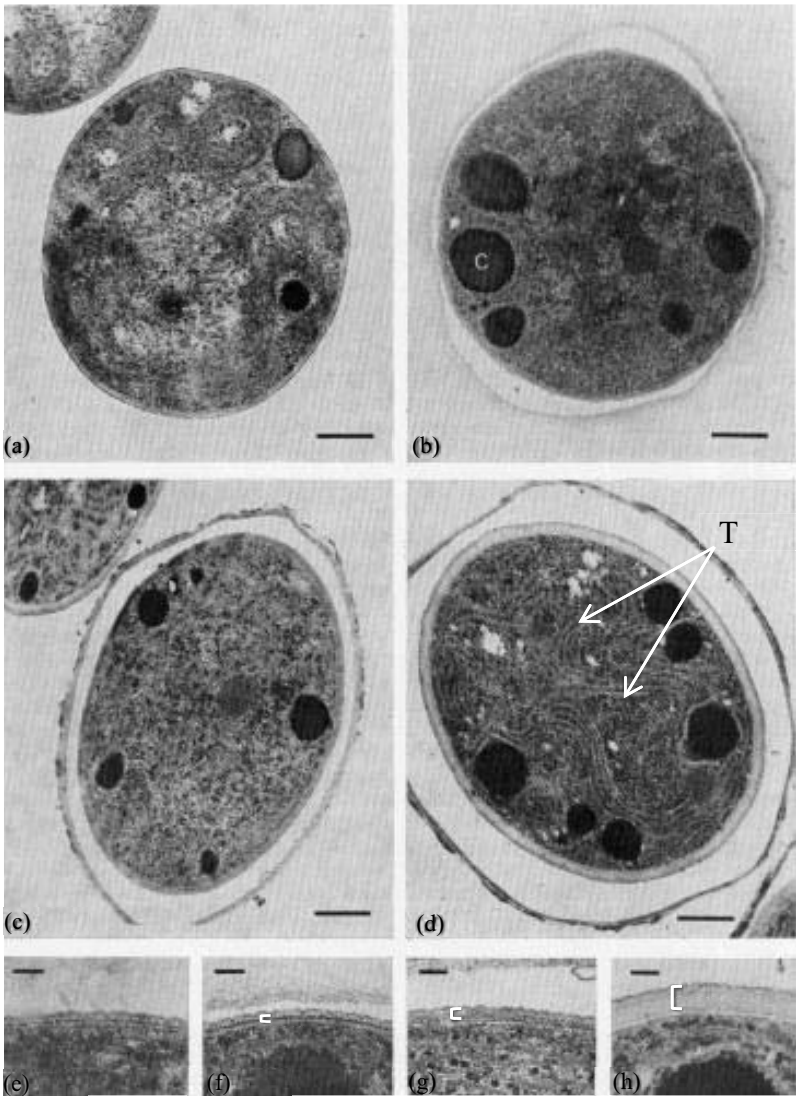

Figure 3 : Illustration des changements morphologiques opérant durant la différenciation d'une cellule végétative de la cyanobactérie *Nostoc* PCC7524 en akinète. (a) cellule végétative, (b) akinète jeune avec accumulation de cyanophycine (C). (c) akinète en maturation, (d) akinète mature, les thylakoïdes (T) sont bien visibles. (e à h): zoom sur les parois cellulaires des figures a à d, respectivement. L'augmentation de l'épaisseur de la paroi cellulaire durant la maturation d'un akinète de *Nostoc* PCC 7524 est due à la couche intermédiaire (indiquée). Echelle: 500nm (a à d) et 100nm (e à h). D'après Sutherland (1979).

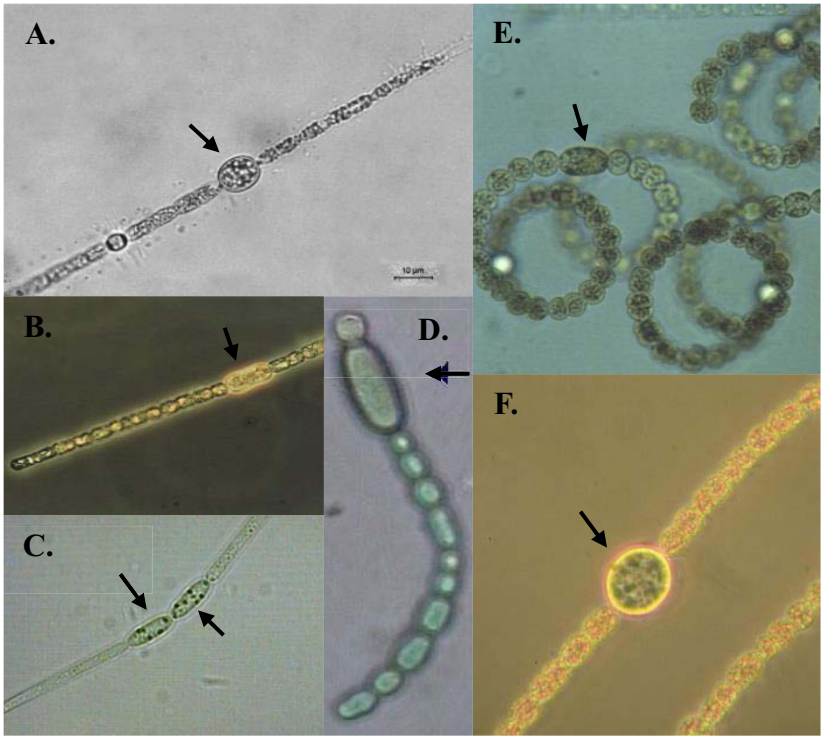

Figure 4: Illustration de la diversité morphologique des akinètes de cyanobactéries. A. *Aphanizomenon ovalispermum* B. *Aphanizomenon flos-aquae* C. *Cylindrospermopsis sp*. D. *Cylindrospermum sp*. E. *Anabaena spiroides* F. *Anabaena macrospora*

Par ailleurs, de faibles températures favoriseraient le développement des akinètes chez la plupart des espèces du genre *Anabaena* (Li*, et al.*, 1997). Les fluctuations de température joueraient un rôle concomitant avec des intensités lumineuses faibles dans la différenciation des spores chez *Cylindrospermopsis raciborskii* (Moore*, et al.*, 2005). Enfin, des milieux carencés en phosphore semblent favoriser le développement des akinètes chez les espèces *Anabaena cylindrica* et *A. circinalis*, alors que des concentrations importantes en ce nutriment stimuleraient celui des *Nostoc* PCC7524 (Sutherland, 1979).

Au cours de leur différenciation, la morphologie et la composition macromoléculaire des akinètes évoluent (Fig.3). Ces spores présentent une diversité morphologique interspécifique importante (Fig.4) ce qui constitue un critère discriminant dans l'identification des espèces cyanobactériennes, au même titre que leur position dans le filament, et par rapport aux hétérocystes. Cependant quelle que soit l'espèce, les akinètes présentent un biovolume plus important que les cellules végétatives. A titre d'exemple, les akinètes matures d'*Anabaena cylindrica* présentent un volume dix fois plus important que les cellules végétatives (Simon, 1977). De plus, les akinètes présentent une paroi plus épaisse que les cellules végétatives et une enveloppe extracellulaire constituée de multiples couches (Sutherland*, et al.*, 1979) (Fig.3). Lors de leur différenciation et de leur maturation, la composition macromoléculaire de ces spores évolue. Aussi, le glycogène et la cyanophycine, polymère de réserves azotées constituées d'aspartate et d'arginine, s'accumulent-ils dans l'akinète. La cyanobactérie *Nostoc* PCC7524 présente des concentrations en cyanophycine 8 fois supérieures à celles des cellules végétatives (Sutherland*, et al.*, 1979). En plus de ce polymère, les akinètes peuvent présenter des concentrations plus élevées en ADN et en protéines que les cellules végétatives. Par exemple, Simon (1977) a rapporté des taux deux fois plus importants d'ADN et dix fois plus importants de protéines chez *Anabaena cylindrica*. Ces fortes concentrations sont à mettre en relation avec la taille importante de ces spores.

Les akinètes jouent un rôle écologique majeur dans la dissémination et la pérennité des espèces cyanobactériennes. Ils ont la capacité de résister à des changements environnementaux importants. Ils peuvent maintenir leur viabilité même dans des environnements froids et secs ; en revanche, ils montrent une grande sensibilité à la chaleur. Les akinètes du genre *Anabaena* détiennent des records de longévité. En effet, Livingstone et Jaworski (1980) ont pu démontrer que des akinètes de ce genre formés 64 ans auparavant étaient encore viables et aptes à la germination. Ainsi, les akinètes revêtent-ils non seulement

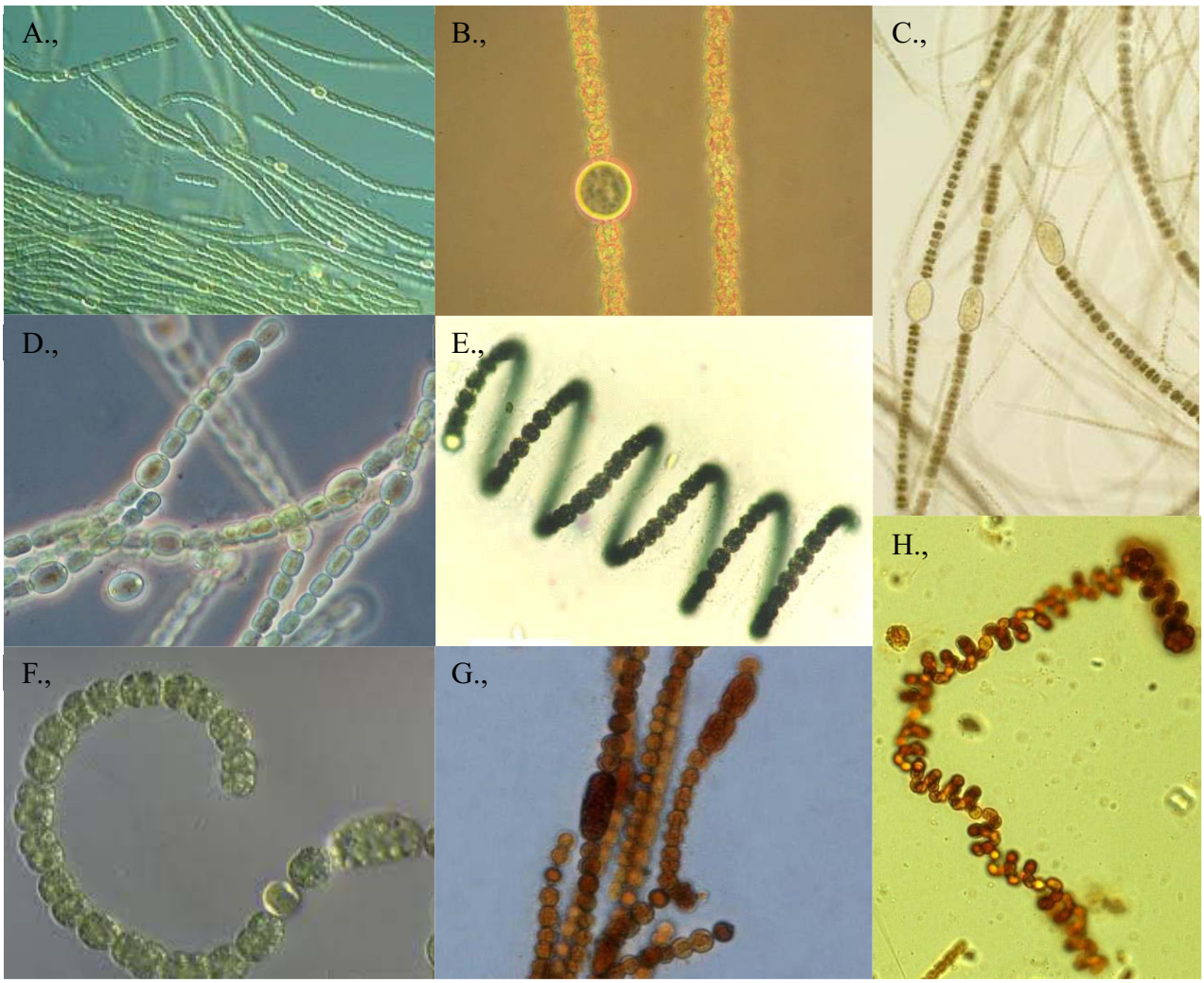

Figure 5: Diversité morphologique du genre *Anabanea* (*Dolichospermum*). A. *A. ambigua* B. *A. macrospora* C. *A. planctonica* D. *A. azollae* E. *A. spiroides* F. *A. flos-aquae* G. *A. affine* H. *A. compactum*

une importance dans les cycles annuels des Nostocales et Stigonematales, mais permettent également une survie à long terme.

4) Le genre Anabaena : Classification, Morphologie et Ecologie

Le genre *Anabaena* appartient à l'ordre des Nostocales (ou section IV de l'I.C.P.N) et à la famille des *Nostocaceae*. Sur la base de la biologie moléculaire, la classification du genre a récemment été revisitée. Depuis 2009, les espèces planctoniques possédant des vésicules à gaz (ex : *A. flos-aquae*, *A. oscillariodes*, *A. macrospora*) sont séparées des espèces typiquement benthiques et forment une entité générique unique (Wacklin*, et al.*, 2009). Ces espèces planctoniques ont été reclassées dans le nouveau genre *Dolichospermum*. Cependant, la nomination générique « *Anabaena* » persiste au sein de la communauté scientifique (Oliver*, et al.*, 2012, Gélinas*, et al.*, 2013). Au cours de cette thèse nous avons choisi de nommer la cyanobactérie impliquée dans la relation hôte-parasite *Anabaena macrospora* (syn. *Dolichospermum macrosporum*).

Le genre *Anabaena lato sensu* présente une diversité morphologique importante (Fig.5). Cependant quelle que soit l'espèce considérée, les cellules végétatives sont sphériques (diamètre: 4-8µm) ou ellipsoïdales (4-8µm de large ; 3-9 de long) et organisées en filaments parfois entourés d'une fine enveloppe mucilagineuse (John*, et al.*, 2002). Toutes les espèces ont la capacité de former des hétérocystes et des akinètes. La place et la forme de ces deux cellules différenciées représentent des critères importants pour la discrimination spécifique. Au sein de ce genre les cellules apicales des filaments ne sont jamais différenciées. Les espèces planctoniques présentent des cellules végétatives possédant des vésicules à gaz, caractère non partagé avec les espèces benthiques. Ces vésicules à gaz confèrent une flottabilité aux espèces planctoniques. De plus, ces structures cylindriques creuses jouent un rôle primordial dans le cycle annuel des Nostocales lors du passage de la phase benthique à la phase planctonique (Fig.2).

Le genre *Anabaena* présente une répartition cosmopolite (Tableau 2). Ainsi, certaines espèces comme *Anabaena lemmermannii* sont retrouvées dans des lacs polaires oligotrophes (Lepisto & Holopainen, 2008). *A contrario*, certaines espèces sont retrouvées sous des conditions subtropicales très eutrophes. En 1991, une efflorescence remarquable d'*Anabaena circinalis* s'est étendue sur près de 1 000 km dans la rivière Barwon-Darling, (Nouvelle-Galles du Sud, Australie) durant près de 2 mois (Bowling & Baker, 1996).

Tableau 2: Répartition géographique de diverses espèces appartenant au genre *Anabaena*.

	Habitat	Répartition géographique							
		Europe	Amérique du nord	Amérique du sud	Afrique	Asie	Australie & Nouvelle Zélande	Arctique	Iles Pacifique
A. ambigua	Eau douce (étang)	8				9,1	6		
A. augstumalis	Soil	1, 3,11					6		
A. azollae	Symbionte de la fougère *Azolla*	2,3,4				12			
A. catenula	Eau douce (étang)	1,2,3,4,5							7
A. circinalis	Eau douce (lac et rivière)	1		13		14			
A. cylindrica	Freshwater (étang)	1,15,16,17	18			19	6		
A. fertilissima	Rizière		20						
A. flos-aquae	Eau douce	1, 3,21, 22	18	13	23	19, 24	6, 25		
A. inaequalis	Eau douce	1, 3	18			19	6,25		
A. lapponica	Eau douce	1,3,27					6	26	
A. macrospora	Eau douce	1,17		14			6,25		
A. planctonica	Eau douce	2,11	18			14, 28	6,25		
A. sphaerica	Sol	2,3,4	18			10	6		
A. spiroides	Eau douce	1,2,11,29	18	13		9, 14,19, 24	6,25		
A. torulosa	Pré salé, eau douce	1,3			31	10	6,25		32
A. variabilis	Eau douce	1,2,3,4,30	16,18	33	34	19	6		
A. verrucosa	Eau douce	1,2,3,4	18				25		

1. (Whitton, *et al.*, 1998)**2.** (Cărăuş, 2002)**3.** (Cărăuş, 2012), **4**. (Cobelas & Gallardo, 1988) **5**. (Ersanli, *et al.*, 2006) **6**. (Day, *et al.*, 1995) **7.** (Sherwood, 2004) **8**. (Bostock, *et al.*, 2007) **9**. (Silva, 1996) **11**. (Täuscher, 2011) **12.** (Mehwish & Aliya, 2005) **13.** (Werner, 2010) **14**. (Pham, *et al.*, 2011)**15**. (Olli, *et al.*, 2005)**16**. (O'Brien, *et al.*, 2006) **18**. (Smith, *et al.*, 2006) **19**. (Hu, 2006) **20**. (Dawes, 1974) **21.** (Nielsen, 2006) **22.** (Täuscher, 2011) **23**. (Thomazeau, *et al.*, 2010) **24**. (Vinogradova, *et al.*, 2000) **25**. (Broady & Merican, 2012) **26.** (Croasdale, 1973) **27** (Spoof, *et al.*, 2006) **28**. (Ramzannejad 2008) **29.** (Willame , *et al.*, 2006) **30.** (Nic Dhonncha & Guiry, 2002) **31.** (Alfinito, 2011) **32.** (Payri & N'Yeurt, 1997) **33**. (Oliveira Filho, 1977) **34**. (Papenfuss, 1968)

Par ailleurs, d'autres espèces du genre *Anabaena* ne sont pas libres et vivent en relation symbiotique. C'est le cas par exemple de la cyanobactérie *Anabaena azollae* (Raja*, et al.*, 2012), symbionte de la fougère aquatique *Azolla*. Cette cyanobactérie est principalement retrouvée dans les rizières des régions chaudes d'Asie, où elle constitue un véritable engrais grâce à ses capacités de fixer le N_2.

II. Les Chytridiomycota

D'un point de vue évolutif, les champignons, au même titre que les cyanobactéries, semblent avoir joué un rôle majeur dans l'apparition des eucaryotes terrestres. En effet, d'après Heckman *et al.,* (2001) l'une des étapes primordiales de la colonisation terrestre des eucaryotes pourrait être la formation de lichens, résultant de l'association symbiotique d'un champignon (appartenant aux *Glomeromycota*) et d'une cyanobactérie unicellulaire. A l'heure actuelle, près d'un cinquième des espèces de champignons connues est lichénisé. Parmi celles-ci, 1500 espèces ont été décrites comme étant impliquées dans des interactions symbiotiques avec des cyanobactéries appartenant principalement au genre *Nostoc* (Rikkinen*, et al.*, 2002). Cependant aucune espèce appartenant au phylum des Chytridiomycota (autrement nommés chytrides) n'a été reportée comme impliquée dans ce type de relation symbiotique. En revanche, les chytrides sont impliqués dans des relations interspécifiques de type parasitaire ou saprophytique. Les chytrides sont des organismes hétérotrophes. Selon une méthode de datation basée sur l'horloge moléculaire, leur apparition est estimée à 1.5 Ga (Heckman*, et al.*, 2001), bien que les premiers fossiles non équivoques ne soient datés que de 450Ma (Krings*, et al.*, 2009) (Fig. 6).

1) Les Chytridiomycota : Une classification fongique sans cesse renouvelée

Les *Chytridiomycota* présentent une paroi chitineuse au cours de leur cycle de vie, ils stockent des carbohydrates comme le glycogène et enfin possèdent la voie de l'acide α-aminoadipique permettant de synthétiser l'amino-acide lysine. Aux vues de ces trois critères, les chytrides sont définis comme des champignons vrais ou Eumycètes et appartiennent au règne des Fungi. Celui-ci se subdivise en un sous- règne, les *Dikarya* (*Ascomycota* et *Basidiomycota*) et cinq phyla : Les *Glomeromycota*, les *Microsporidia*, les *Blastocladiomycota*, les *Neocallimastigota* et les *Chytridiomycota* (Hibbett*, et al.*, 2007).

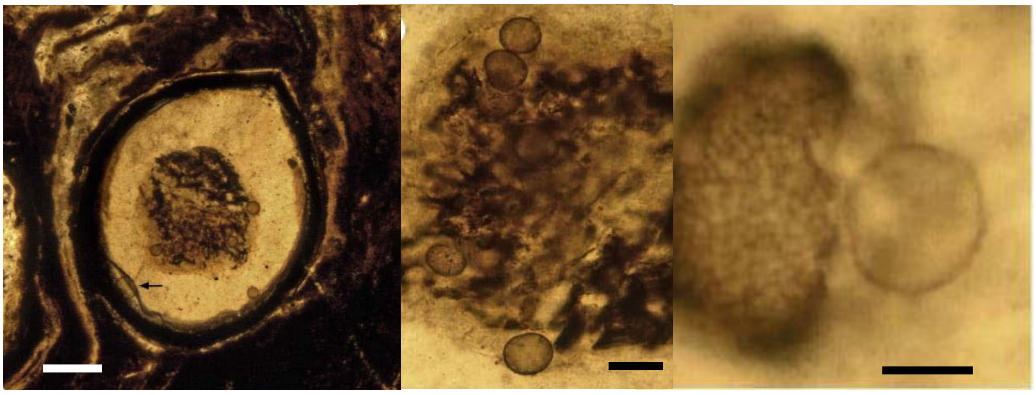

Figure 6: Chytrides fossiles datant du Visséen. A. Mégaspore de *Sublagenicula nuda-type* (Lycophyte) colonisé par un chytrid au niveau de la paroie cellulaire, échelle=150 µm; B. Détail de l'intérieur de la mégaspore, échelle= 50µm; C. Spore de fougère présentant un sporange de chytride attaché à sa surface, échelle =10 µm. D'après Krings *et al.,* 2009

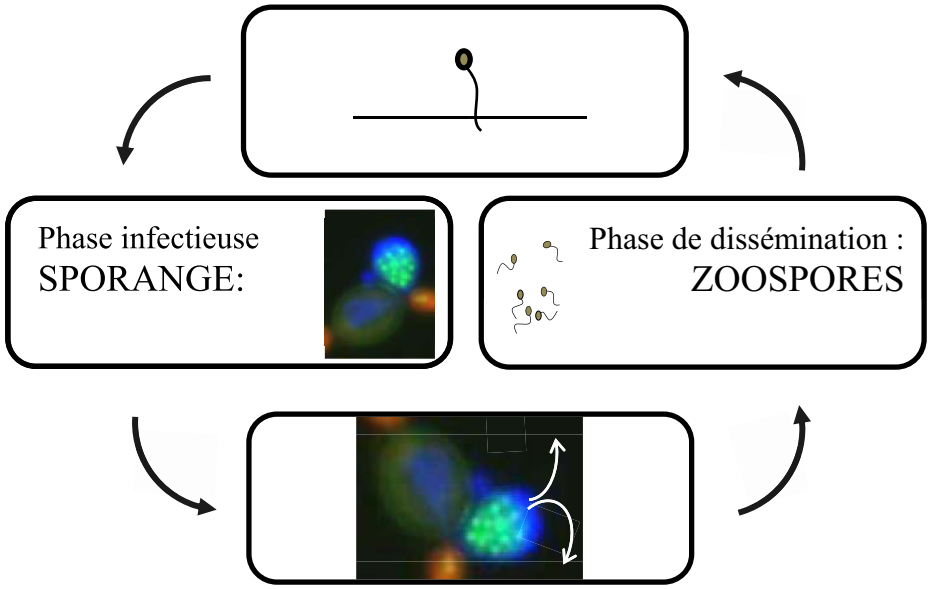

Figure 7: Cycle de vie des chytrides parasites du phytoplancton dans les écosystèmes lacustres

Jusqu'en 2011, les Chytridiomycota étaient considérés comme la branche la plus basale de la classification phylogénétique des Fungi. Les travaux menés par Jones *et al.,* (2011) ont changé cette vision en mettant en évidence une nouvelle clade nommée Cryptomycota, considérée à l'heure actuelle comme la branche la plus ancestrale du règne des Fungi. Précédemment basée sur des critères morphologiques de leur thalle[1] (Sparrow, 1960), la classification mycologique évolue désormais au rythme des nouvelles données moléculaires et est en perpétuelle construction/destruction de nouveaux ordres, classes ou phyla. (Schüßler*, et al.*, 2001, James*, et al.*, 2006, James*, et al.*, 2006, Jones*, et al.*, 2011). A l'heure actuelle, le phylum des Chytridiomycota est composé de six ordres distincts : Chytridiales, Spizellomycetales, Rhizophydiales, Rhizophyctidales, Lobulomycetales et Cladochytriales (Powell & Letcher, 2012), dont il a été décrit environ 1000 espèces (James*, et al.*, 2006). Quel que soit l'ordre considéré, les Chytridiomycota présentent des caractéristiques communes et propres au phylum.

2) *Les Chytridiomycota : Morphologie, Ecologie et Cycle de vie*

Les chytrides sont des organismes présentant deux phases distinctes dans leur cycle de vie : une phase de dissémination, nommée zoospore, et une phase fixée (parasitaire ou saprophytique) nommée sporange (Fig. 7). Les thalles sont les caractères morphologiques les plus visibles du cycle de vie des chytrides. Le thalle peut produire, en plus des rhizoïdes, un seul ou plusieurs sporange(s), le chytride sera alors qualifié respectivement, de chytride monocentrique ou polycentrique. Par ailleurs, les chytrides peuvent se développer au sein (endobiotique), ou à l'extérieur de leur hôte (épibiotique) (Powell & Letcher, 2012). Les sporanges, qui peuvent être operculés (un opercule permet la libération des zoospores) ou inoperculés (les zoospores sont libérées au travers de la paroi du sporange devenue poreuse) sont le siège de la reproduction asexuée (R.A). En effet, la production de multiples zoospores uninuclées résulte de nombreuses mitoses successives où l'intégralité du contenu cytoplasmique est alors transformée (Berger*, et al.*, 2005). Cependant, lorsque les conditions environnementales deviennent défavorables, la mise en place d'une reproduction sexuée (R.S) s'effectue. Ce mode de reproduction, démontrée chez *Zygorhizidium planktonicum*, reste encore méconnu chez d'autres espèces comme *Rhizophydium planktonicum* (Ibelings, *et al.*, 2004). Les zoospores qui résultent soit de la R.A soit de la R.S mesurent de 2 à 5µm selon les

[1] Un thalle est un appareil végétatif ne possédant ni feuille, ni racine, ni tige

espèces (Kagami*, et al.*, 2004). Lors de leur dissémination, les zoospores ne se nourrissent pas, et tirent leur énergie de leurs réserves lipidiques (Suberkropp & Cantino, 1973). Piotrowski *et al.,* (2004) ont montré que 95% des zoospores de *Batrachochytrium dendrobatidis* cessaient toute activité au bout de 24h, et ne nageaient pas plus de deux cm en moyenne avant de s'enkyster, ce qui suggère que la densité de cellules hôtes est un paramètre primordial dans l'infection parasitaire.

Comme dit précédemment, les chytrides peuvent interagir avec d'autres organismes *via* le parasitisme ou le saprophytisme. Grâce à leur flagelle postérieur propulseur, les zoospores sont parfaitement adaptées aux milieux aquatiques. Ainsi les chytrides sont retrouvés dans des étangs (Gromov*, et al.*, 1999, Shin*, et al.*, 2001), des lacs (Lefèvre*, et al.*, 2012, Rasconi*, et al.*, 2012) et des rivières (Richards-Zawacki, 2010). Bien qu'hautement liés au milieu aquatique pour leur phase de dissémination, les chytrides sont retrouvés dans de nombreux habitats terrestres tels des sols forestiers, agricoles, ou plus surprenant, désertiques (Gleason*, et al.*, 2010). En réalité, une fine pellicule d'eau suffit à ces spores pour se déplacer. Dans le milieu terrestre, les chytrides peuvent être parasites de plantes vasculaires (Barr, 2001) ou saprotrophes et dégrader des matières réfractaires telles que la chitine, la kératine, ou l'exine (composé formé de sporopollénine et de cellulose formant la paroi du pollen) (Masclaux*, et al.*, 2011). Par ailleurs, les chytrides sont plus connus au travers d'une espèce, *Batrachochytrium dendrobatidis.* Seule espèce parasite de vertébrés, *B. dendrobatidis* est considérée comme l'un des facteurs majeurs du déclin des populations d'amphibiens (Berger*, et al.*, 2005). Dans les environnements pélagiques, les chytrides ont un rôle très important puisqu'ils seraient l'un des facteurs biotiques responsable de la succession phytoplanctonique (Van Donk, 1989). Par ailleurs, au travers de leur phase de dissémination, assimilable par le zooplancton ils constitueraient un véritable lien entre le compartiment phytoplanctonique et les niveaux trophiques supérieurs (Kagami*, et al.*, 2007). Ces aspects sont plus largement développés dans la 2ème partie de la synthèse bibliographique II-2-3.

2ème partie- Les efflorescences de cyanobactéries

I. Les cyanobactéries : des microorganismes « classés nuisibles »

Les écosystèmes aquatiques sont soumis à un forçage anthropique extrêmement important. Les apports en engrais par l'agriculture intensive, tout comme les apports en phosphates d'origine anthropique (déchets ménagers, lessives…), ont augmenté de manière inconsidérée les concentrations en nutriments, conduisant ainsi à une eutrophisation des écosystèmes aquatiques. Cette eutrophisation n'est pas sans conséquence sur la biologie des écosystèmes. L'un des premiers compartiments biotiques impactés est le phytoplancton. N'étant plus limités en nutriments, ces producteurs primaires voient leur biomasse très fortement augmenter dans les écosystèmes aquatiques anthropisés (Smith*, et al.*, 1999). Les augmentations de biomasse les plus visibles et les plus étudiées se font sous forme de véritables efflorescences phytoplanctoniques. Celles produites par les cyanobactéries sont parmi les plus spectaculaires (Huisman*, et al.*, 2005). Ces proliférations cyanobactériennes, ou « blooms » cyanobactériens, sont principalement dues aux espèces appartenant aux genres planctoniques *Microcystis*, *Cylindrospermopsis*, *Planktothrix* et *Anabaena.*

En fonction de l'espèce incriminée dans une prolifération cyanobactérienne, la répartition spatiale des cellules diffère. Ainsi, les blooms des espèces *Microcystis* et *Anabaena* forment d'importantes écumes vertes flottant à la surface de l'eau, vraisemblablement dûes à la présence de vésicules à gaz chez les espèces de ces genres (Oliver*, et al.*, 2012). Au contraire, les espèces du genre *Cylindrospermopsis* ou l'espèce *Planktothrix agardhii* présentent une répartition plus diffuse dans l'épilimnion[2]. Enfin, des espèces cyanobactériennes telles que *Planktothrix rubescens,* adaptées aux intensités lumineuses très faibles forment des blooms dans les couches d'eau plus profondes des lacs, notamment au niveau du métalimnion[3] (Sotton*, et al.*, 2011).

[2] L'épilimnion correspond à la couche d'eau supérieure d'un écosystème lacustre lorsque celui-ci est stratifié. En saison estivale, il présente les températures les plus élevées dans la colonne d'eau.

[3] Le métalimnion correspond à la couche d'eau sous-jacente à l'épilimnion d'un système lacustre stratifié, où se situe notamment la thermocline (zone de décroissance rapide de la température avec la profondeur).

Dans un contexte de changement global, les nombreux modèles élaborés tendent à montrer une augmentation de la durée et de la fréquence des blooms cyanobactériens dans de nombreux écosystèmes (Huber*, et al.*, 2008, Paerl & Huisman, 2009, Elliott, 2011). Ceci est loin d'être anecdotique car les efflorescences cyanobactériennes sont connues pour avoir d'importantes conséquences néfastes non seulement sur le plan récréatif, mais aussi sur les plans écologiques et économiques. Du point de vue écologique, les cyanobactéries déséquilibreraient le fonctionnement des réseaux trophiques. En effet, la plupart des espèces capables de former des blooms cyanobactériens sont considérées comme de véritables impasses trophiques de par leur importante taille et leur morphologie (coloniale et filamenteuse) qui constituent un véritable refuge contre la prédation. D'autre part, certaines espèces peuvent synthétiser des toxines, dont le rôle est encore incertain, mais qui pourraient avoir un effet allélopathique (Singh*, et al.*, 2001) voire mortel pour certains organismes prédateurs ou compétiteurs (Schlegel*, et al.*, 1998, Rohrlack*, et al.*, 2001, Hansson*, et al.*, 2007). La perte de diversité phytoplanctonique induite lors d'un bloom cyanobactérien provoque une réaction en chaine pouvant aboutir à une perte de diversité ou à une mortalité piscicole importante. En effet, les phénomènes de *fish-killing* sont provoqués par l'anoxie nocturne due à une importante activité respiratoire des cyanobactéries. Il faut ajouter à cela les fermetures de baignade liées aux risques sanitaires dues aux cyanotoxines. Ainsi, les conséquences écologiques et sociétales de l'eutrophisation des écosystèmes aquatiques sont à l'origine d'importantes pertes économiques évaluées à 2.2 milliards de dollars par an aux Etats-Unis (Dodds*, et al.*, 2008). Au vu de l'importance écologique et économique que représentent les efflorescences cyanobactériennes, une connaissance exhaustive des facteurs de régulation est nécessaire.

II. Facteurs influençant la dynamique des « blooms » cyanobactériens

1) Des facteurs abiotiques bien connus

La dynamique des blooms cyanobactériens est fortement influencée par les facteurs abiotiques. Il apparait clairement que la température joue un rôle majeur dans la dominance et le déclin des blooms cyanobactériens (Paerl & Huisman, 2008). Elle impacte directement les métabolismes de toutes les entités biologiques. Les cyanobactéries présentent des optima de

croissance à des températures beaucoup plus élevées que celles des diatomées ou des chlorophycées (Paerl & Huisman, 2009), expliquant leur dominance lors de périodes estivales. De plus, la température conditionne les mouvements de masses d'eau au sein des écosystèmes aquatiques. Ainsi, des températures élevées provoquent une stratification de la colonne d'eau. Lors de cette stabilité physique, les cyanobactéries sont particulièrement avantagées par rapport aux autres espèces planctoniques (Jöhnk, *et al.,* 2008). En effet, en plus des vésicules à gaz, les cyanobactéries utilisent les carbohydrates (principalement sous forme de glycogène) produits et stockés lors du processus photosynthétique, comme de véritables ballasts (Brookes*, et al.*, 1999). Grâce à ces deux mécanismes physiques, les cyanobactéries peuvent réguler leur flottabilité, se maintenir dans la zone euphotique[4] et épilimnique, et éviter ainsi la sédimentation. Par ailleurs, les concentrations en nutriments disponibles, facteurs primordiaux dans la dynamique phytoplanctonique, sont elles aussi en partie conditionnées par la température. En effet, la stratification de la colonne d'eau empêche les flux de nutriments des zones profondes vers la zone euphotique où ils deviennent rapidement limitants (Sarmiento*, et al.*, 2004). Grâce à leur capacité de fixation de l'azote atmosphérique, les cyanobactéries telles que les nostocales peuvent s'affranchir de cette limitation. En revanche, quand les températures chutent, la stabilité de la colonne d'eau est rompue et le lac est alors engagé dans un processus de brassage. Lors de ces mouvements d'eau, les nutriments sont renouvelés et l'ensemble des cellules phytoplanctoniques ont de nouveau accès à la zone euphotique grâce aux mouvements de convection. Les cyanobactéries perdent alors leur avantage au profit de populations phytoplanctoniques adaptées aux températures plus faibles et aux conditions de brassage (Visser*, et al.*, 1996).

2) Les facteurs biotiques : Des facteurs établis aux facteurs moins étudiés

Les facteurs abiotiques conditionnent la dynamique cyanobactérienne. Les nombreuses études menées sur le sujet ont pu établir clairement l'influence de plusieurs paramètres abiotiques, notamment ceux discutés précédemment. Cependant, il est certain qu'à ces conditions environnementales s'ajoutent les facteurs biotiques. Cette section a fait l'objet d'un article de synthèse en cours de soumission.

[4] La zone euphotique d'un lac, ou d'un océan s'étend jusqu'à une profondeur à laquelle l'intensité lumineuse résiduelle correspond à 1 % de celle en surface.

Article 1

Microbial players involved in the decline of filamentous and colonial cyanobacterial blooms with a focus on fungal parasitism

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Running title: *Microorganisms involved in cyanobacterial bloom decline*

Summary

In the forthcoming decades, it is widely believed that the dominance of colonial and filamentous bloom-forming cyanobacteria (e.g *Microcystis*, *Planktothrix*, *Anabaena* and *Cylindrospermopsis*) will increase in freshwater systems as a combined result of anthropogenic nutrient input into freshwater bodies and climate change. Whilst the physico-chemical parameters controlling bloom dynamics are well known, the role of biotic factors remains comparatively poorly studied. Morphology and toxicity often - but not always - limit the availability of cyanobacteria to filter feeding zooplankton (e.g. cladocerans). Filamentous and colonial cyanobacteria are widely regarded as trophic dead-ends mostly inedible for zooplankton, but substantial evidence shows that some grazers (e.g. copepods) can bypass this size constraint by breaking down filaments, making the bloom biomass available to other zooplankton species. A wide range of algicidal bacteria (mostly from the *Alcaligenes*, *Flavobacterium*/*Cytophaga* group and *Pseudomonas*) and viruses (*Podo-, Sipho-,* and *Myo-viridae*) may also contribute to bloom control, via their lytic activity underpinned by a diverse array of mechanisms. Fungal parasitism by the Chytridiomycota remains the least studied. Whilst each of these biotic factors has traditionally been studied in isolation, emerging research consistently point to complex interwoven interactions between biotic and environmental factors.

Introduction

During the past century, inputs of nutrients such as nitrogen and phosphorus into freshwater ecosystems have increased due to human activity, resulting in widespread eutrophication (Tilman, 1999; Paerl, 2006). This phenomenon has often been related to the seasonal proliferation of cyanobacteria that can outcompete and dominate other phytoplankton assemblages (Dokulil and Teubner, 2000; Paerl and Fulton, 2006). Climatic changes (e.g global warming, inter-annual temperature changes) and associated climatic oscillations (e.g North Atlantic Oscillation (NAO), Arctic Oscillation (AO) (Báez *et al.*, 2014)) affect the phytoplankton community structure as well. Increases in temperature with lengthened stratification periods and increased nutrient inputs, have led the scientific community to forecast increased dominance of harmful cyanobacteria (e.g *Microcystis*, *Planktothri*x, *Anabaena* and

Cylindrospermopsis) in various aquatic ecosystems (Huber *et al.*, 2008; Paerl and Huisman, 2008; Paerl and Huisman, 2009; Elliott, 2011; Kosten *et al.*, 2012). As a result, the adverse economic impact of cyanobacterial blooms is expected to worsen, affecting recreational and angling activities, lake property values, and increasing the cost of drinking water treatment. It has been estimated that the annual cost of eutrophication was approximately $2.2 billion (USD) in US freshwater ecosystems (Dodds *et al.*, 2008). Another source of concern is the resulting loss of phytoplankton biodiversity, and the subsequent impoverishment of zooplankton community (Ramirez Garcia *et al.*, 2002) that could potentially impact fish diversity (Romo and Villena, 2005). Indeed, cyanobacterial blooms are often mono specific and cyanobacteria might represent up to 99% of the total phytoplankton biomass that disrupt food web process due to three main factors: morphology of cyanobacteria (De Bernardi and Giussani, 1990), their poor nutritional quality for zooplankton (Elert *et al.*, 2003) and finally their capacity to produce toxins (Sivonen, 1996). Like all phytoplankton, cyanobacterial growth is mainly governed by both bottom-up (nutrients concentration, light intensity, temperature…) and top-down (predation, parasitism) control. During the last decades, abiotic factors such as the nutrients (N and P) concentration, their ratio, temperature and mixing of the water column, were shown to partly drive both the onset and the decline of cyanobacterial blooms (Visser *et al.*, 1996; Oliver and Ganf, 2002; Paterson *et al.*, 2002; Havens *et al.*, 2003; Paerl and Huisman, 2008). Here, we review the main microbial- and viral-mediated top-down regulation factors of freshwater cyanobacterial blooms, with an emphasis on parasitism. Whilst generally overlooked, the role of parasites is discussed in proportion to the relative contribution of protozoan and metazoan predators to food web dynamics. Non microbial biological drivers (fishes and mollusks) contributing to the decline of cyanobacterial blooms are comparatively better covered in the literature. Interested readers are referred to the papers by Xie and Liu (2001), Lance *et al.,* (2006), Lu *et al.,* (2006) and White and Sarnelle, (2014).

Biotic factors promoting the decline of cyanobacterial blooms

Grazing

Zooplankton communities able to graze on cyanobacteria encompass four major groups: rhizopods, ciliates, rotifers and crustaceans. For several decades, studies have investigated trophic interactions between cyanobacteria and diverse zooplankton communities such as testate amoebae (Nishibe *et al.*, 2004; Mizuta *et al.*, 2011), rotifers (Starkweather and Kellar, 1983; Van Wichelen *et al.*, 2010; Kâ *et al.*, 2012), ciliates (Canter *et al.*, 1990; Audrey *et al.*,

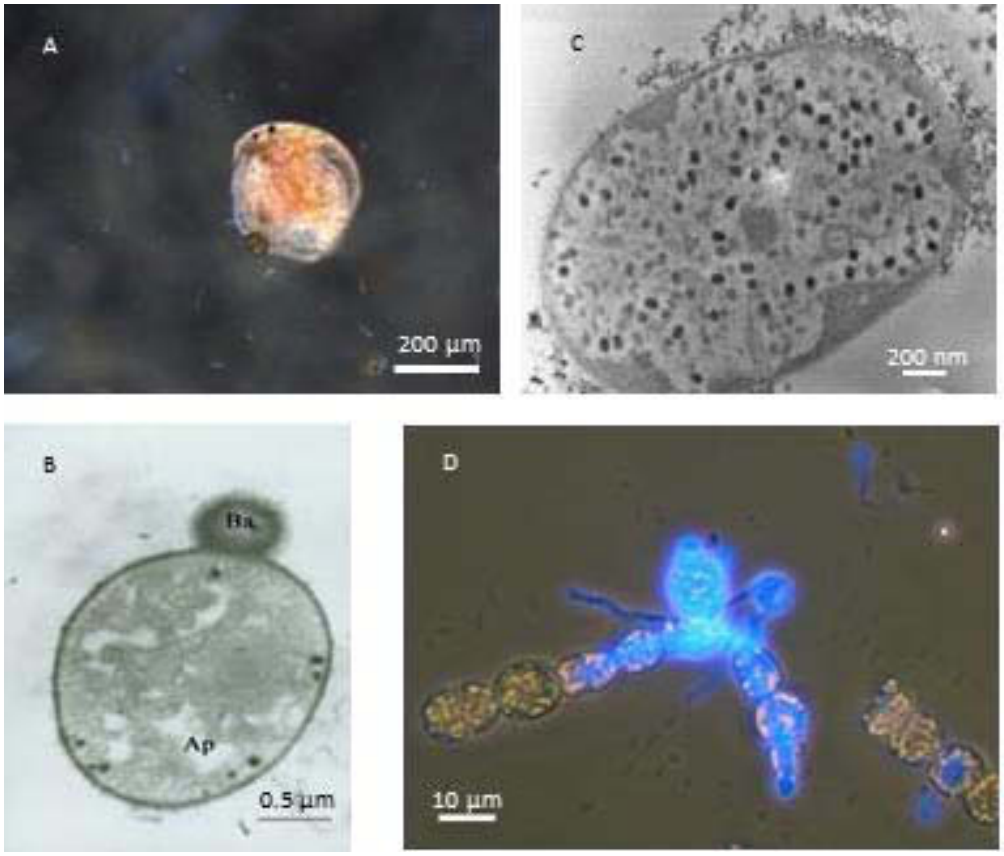

Figure 1: Examples of grazing (A), lysis (B-C) and parasitism (D) of cyanobacteria. Cladoceran grazing on *Microcystis sp.* (A). Bacterium DC22 (Ba.) attached to the *Aphanizomenon flos-aquae* cell (Ap) (B). *Anabaena macrospora* infected with numerous tailed viruses (C). Mechanistic fragmentation of *Anabaena macrospora* filament by Chytridiomycota (D). (Pictures taken by Gerphagnon M. (A, D), Shunyu *et al.,* 2006 (B) and Colombet J. (C))

2012), cladocerans (Lampert, 1987; Boon *et al.*, 1994; Gustafsson and Hansson, 2004; Oberhaus *et al.*, 2007) (Fig. 1A) and copepods (Haney, 1987; DeMott *et al.*, 1991; Rollwagen-Bollens *et al.*, 2013) (Supplemental Table S1). Although it is clearly established that grazers influence the phytoplankton succession (Sommer *et al.*, 1986; Sommer *et al.*, 2012) and can be responsible for more than 50% of phytoplankton loss in some cases, there are conflicting reports on how zooplankton can contribute to the decline of cyanobacteria.

The cyanobacterial morphology and toxicity have been extensively studied and described as putative limitations of the top-down control exerted by zooplankton on cyanobacteria (Ger *et al.*, 2014). Generally, prey morphology is the most important factor influencing zooplankton grazing. Colonial or filamentous cyanobacterial forms can interfere mechanically with the filtering apparatus of zooplankton by sealing it, implying that zooplankton may not be able to control all cyanobacterial blooms (Paerl and Otten, 2013; Rollwagen-Bollens *et al.*, 2013). Nevertheless, morphological and behavioural adaptations allow grazers to bypass this size constraint. Smaller species possess smaller opening carapace that cannot engulf large filaments or colonies, and are thus less affected than larger grazers. De Mott *et al.,* (2001) highlighted important differences in feeding and growth rate between grazers, relative to their size. *Daphnia cucullata*, the smallest species, demonstrated the best efficiency to grow on filamentous species (*Oscillatoria limnetica* and *Prochlorothrix hollandica*) whereas two larger cladoceran species failed. Some field observations suggest that filamentous cyanobacterial blooms induce a shift from large to small filter feeders (Gliwicz, 1977). In contrast to generalists, copepods can select their food. For example, *Notodiaptomus iheringi* copepods consume cyanobacteria, but they were more selective for smaller colonies (Ghadouani *et al.*, 2003; Panosso *et al.*, 2003). On the other hand, copepods showed the ability to break the large filaments to a smaller size making them more palatable for them and other zooplankton species (Bouvy *et al.*, 2001; Kâ *et al.*, 2012). Moreover, through bottom up factor, as phosphate depletion, the growth of the filaments and consequently their size could be reduced (Jacquet *et al.*, 2014) and thus promote the grazing pressure exerted by *Daphnia* onto *Planktothrix rubescens* (Oberhaus *et al.*, 2007). As well as allowing efficient grazing, such breakage may physiologically affect the cyanobacteria, especially heterocystous species. Chan *et al.,* (2004) have shown that grazing significantly suppressed nitrogen fixation and cyanobacterial growth, by reducing the filament size of *Anabaena* spp.

Otherwise, Van Wichelen *et al.* (2010) established that beyond the size of cyanobacterial colonies, their morphotype can play an important role in grazing behaviour. Actually, despite

Microcystis viridis morphotype presented the smallest colony size, this later was not grazed by amoeba, whereas the *M. aeruginosa* morphotype was. Consequently, the cyanobacterial population switched rapidly from grazing-sensitive (*M. aeruginosa* morphotype) to grazing-resistant (*M. viridis* morphotype) *Microcystis* genotypes in Westveld Pond, Belgium. Regarding that a major difference between both morphotypes was the thick of their mucilage matrix (*M. viridis* presenting the densest, extended mucilage); the authors suggested that the thick mucilage layer of *Microcystis* colonies as well as its biochemical composition may be real physical and chemical barriers for the amoeba grazing. Moreover, some compounds excreted by *M. viridis* seem to play an important role in the grazing rate of amoebae (Van Wichelen *et al.*, 2012). Recently, Urrutia-Cordero *et al.* (2013) showed that cyanotoxins can alter the cytoskeleton structure of the grazer *Acanthamoeba castellanii*, which leads to its death. Likewise, microcystin is lethal for a wide range of *Daphnia* species (Rohrlack *et al.*, 2001) and copepods (DeMott *et al.*, 1991; Ger *et al.*, 2010). In a study encompassing six lakes in the southernmost part of Sweden, Hansson *et al.* (2007) correlated a decrease in the biomass of cladocerans and copepods with increasing microcystin concentrations ranging from 0 to 30µg.L^{-1}. Additionally, adult cladocerans which were not killed by microcystin were reduced in size and the biomass of juvenile *Daphnia* was diminished. Smaller zooplankton species (cyclopoid copepods, *Bosmina* and rotifers) were not impacted by microcystin, which is consistent with a study showing that the smallest species of cladocerans and copepods were not, or only slightly, affected by cyanobacteria (Guo and Xie, 2006). Moreover, zooplankton communities from eutrophic habitats exhibit physiological adaptations to cyanotoxins, such as detoxification capability (Schwarzenberger *et al.*, 2012; Kuster and von Elert, 2013). For example, following prior exposure to toxic cyanobacteria, *Daphnia pulicaria* and *D. magna* strive even at high concentration of microcystins (Sarnelle and Wilson, 2005; Sarnelle *et al.*, 2010). Furthermore, this tolerance could be transferred to offspring via maternal effects (Gustafsson *et al.*, 2005). Some other grazers (e.g. *Ochromonas* sp.) also appear to efficiently graze irrespective of both microcystin content and prey morphology (single cells, colonies or filaments) (Van Donk *et al.*, 2009).

After more than 20 years of research, interactions between cyanobacteria and zooplankton are still not resolved. Overall, there is no general rule regarding the capacity of grazers to control cyanobacterial blooms. It is clear however, that herbivores can alter the structure of cyanobacterial populations. This top down control and the inter-relationship with

cyanobacterial morphology and toxicity are dependent on the specific species pairing (Wilson *et al.*, 2006; Lemaire *et al.*, 2012).

Lysis by heterotrophic bacteria

The great majority of bloom-forming cyanobacteria (*Anabaena*, *Aphanizomenon*, *Microcystis*) are often closely associated with other microorganisms, especially heterotrophic bacteria (Liu *et al.*, 2014). Mutualistic organic matter, energy, oxygen, nitrogen and phosphorous exchanges between these prokaryotes have been reported in the phycosphere (Paerl and Pinckney, 1996; Dziallas and Grossart, 2012). However, some heterotrophic bacteria, far from being involved in a mutualistic relationship, lyse the cyanobacteria as a food resource. Most cyanobacteriolytic bacteria belong to the genera *Alcaligenes,* the *Flavobacterium/Cytophaga* group and *Pseudomonas* (Yamamoto *et al.*, 1993). Bacterial lysis appears to function in three main ways: penetration into the host cell (Caiola and Pellegrini, 1984), cell to cell contact (Shunyu *et al.*, 2006; Gumbo and Cloete, 2013) or most often production of extracellular compounds (Choi *et al.*, 2005; Mu *et al.*, 2007) such as peptides, proteins, amino acids, or antibiotics which may or may not, be host specific (Gumbo *et al.*, 2008). Caiola and Pellegrini (1984) reported for the first time the penetration of *Bdellovibrio*-like bacteria into host cells of *Microcystis aeruginosa* in natural populations (Fig. 1B). The *Bdellovibrio-like* bacteria were found in *M. aeruginosa*, but not in *Microcystis wesenbergii*, suggesting a strict host-specificity. In contrast, Shunyu, *et al*, (2006) found that *Bacillus cereus* induces lysis by cell-to-cell contact on a wide range of cyanobacteria (*Microcystis viridis*, *Microcystis wesenbergii*, *Microcystis aeruginosa*, *Oscillatoria tenuis*, *Nostoc punctiforme*, *Anabaena flos-aquae*, *Spirulina maxima*), as well as chlorophyceae (*Chlorella ellipsoidea*, *Selenastrum capricornutum*).

Most studies investigating the algicidal bacterial activities were conducted under laboratory conditions and only few studies have surveyed the co-dynamics of cyanobacteria and algicidal bacteria in natural conditions (nicely discussed in Ren *et al.,* 2010). In the eutrophic Lake Brome (Quebec), Rashidan and Bird (2001) investigated two pairs of cyanobacteria/bacteria (*Anabaena sp.*/*Cytophaga* strain C1 and *Synechococcus cedorum*/*Cytophaga* strain C2) coupled with measures of the algicidal activity of these two bacterial strains under laboratory conditions. In the field, the couple *Anabaena sp* /*Cytophaga*

strain C1 exhibited typical prey/predator dynamics. The cyanobacterial bloom decline coincided with the maximum of lytic bacteria abundance, suggesting a key role of them in the decline. However, some lytic bacteria studied in the laboratory may prove to have different roles under natural conditions. For example, Ren *et al.,* (2010)*,* showed under laboratory conditions that *Pseudomonas aeruginosa* R219 (*P.a* R219, isolated from Lake Tai (China) during a *Microcystis aeruginosa* bloom) could lyse up to 90% of *Microcystis* cells. However, when following both *M. aeruginosa* and *P.a* R219 dynamics through molecular biology in Lake Tai, Zhang *et al.,* (2012) could not find any significant correlation between cyanobacterial decline and the abundance of *P.a* R219. During this survey, *P.a* R219 acted as a simple decomposer rather than as a key element of the decline of *M. aeruginosa*. Choi *et al.* (2005) suggested that non-obligate predators (i.e. *Cytophaga, Mixobacteria*) only use host cells as a food resource when nutrients become depleted. These inconsistent results could originate from a concentration effect. Zhao *et al.* (2012) studied the effects the algicidal substances excreted by *Bacillus cereus* L7 on *Anabaena flos-aquae* growth. The authors indicated that a high concentration of algae-lytic products ($0.5 mg.l^{-1}$) inhibited the cyanobacterial growth whereas exposure to a ten-fold lower concentration increased growth, chlorophyll a, protein and phycobiliprotein contents.

Viral lysis

Viruses are the most abundant biological entities in aquatic environments, with average abundances fluctuating between 10^7 and 10^8 virus particles mL^{-1} in marine and freshwater systems, respectively (Suttle, 2005; Wilhelm and Matteson, 2008). Most are prokaryotic viruses, in which cyanophages (viruses attacking cyanobacteria) appear to be ubiquitous in aquatic ecosystems where they occur at ca. 10^6 viruses.mL^{-1} (Suttle, 2000). Like other bacteriophages, cyanophages interact with their hosts by two major pathways: the lytic and the lysogenic cycles. The former results in the host lysis, whilst the latter is a stable infection where the viral DNA is integrated into the host genome with no damage to the host cell, until environmental factors induce the lytic pathway (Suttle, 2000). Lysogeny is responsible for the well-known lateral gene transfer within bacterial populations (Suttle, 2005; Rohwer and Thurber, 2009 ; Lang *et al.*, 2012). Until recently, all known cyanophages infecting filamentous or colonial cyanobacteria belonged to one of the three tailed bacteriophage families: *Podoviridae*, *Siphoviridae*, (Fig. 1C) and *Myoviridae*. These viruses belong to the order Caudovirales, and can be morphologically differentiated by their tail, which can be short

(*Podoviridae*), or long (*Siphoviridae* and *Myoviridae*), contractile (*Myoviridae*) or not (*Podoviridae* and *Siphoviridae*). A different group was reported by Deng and Hayes (2008), who isolated a filamentous cyanophage from the Cotswold Water Park, U.K., that is capable of infecting the three major freshwater bloom-forming cyanobacteria *Anabaena*, *Microcystis* and *Planktothrix*. In 2009, Gao *et al.,* reported for the fisrt time a non-tailed virus infecting the filamentous cyanobacteria *Planktothrix* (Supplemental Table S2). For interested readers, McDaniel (2011) gives an historical perspective on cyanophages in both freshwater and marine habitats.

The general ability of viruses to greatly reduce the biomass or the photosynthetic activity of cyanobacteria is now well established (Hewson *et al.*, 2001; Tucker and Pollard, 2005). In an investigation of *Cylindrospermopsis raciborskii* (a filamentous, potentially toxic cyanobacterium isolated from Lake Samsonvale, Queensland, Australia), Pollard and Young (2010) reported that viral lysis reduced the abundance of cyanobacteria by 86% in five days. The lysis of the population is not always exhaustive, as was showed on *Microcystis aeruginosa* (Yoshida *et al.*, 2006). A likely explanation is the virus host specificity, as the cyanophage isolated in this study (Ma-LMM01) only caused lysis on one *M. aeruginosa* strain among the 16 genotypes and other cyanobacterial species tested. Importantly, most studies highlight a larger impact of cyanophages in laboratory conditions compared to the field, with differences as high as 90% and 3% biomass decrease, respectively (Yoshida *et al.*, 2006; Pollard and Young, 2010). Such discrepancies might at least partially result from technical artefacts, because the quantification methods used (i.e RT-qPCR or transmission electron microscopy) are prone to underestimate infection prevalence in natural populations (Yoshida *et al.*, 2010). Yet, other methods developed on picocyanobacteria, which directly estimate the viral-mediated mortality of cyanobacteria (e.g. as the modified dilution method by Evans *et al.* (2003)), also revealed considerable variation in the contribution of viral lysis to cyanobacterial mortality, both in marine (Baudoux *et al.*, 2008) and freshwater (Parvathi *et al.*, 2014) habitats. To our knowledge this method has never been applied on colonial or filamentous cyanobacteria.

Additionally, viruses may play a major role in the dispersion of their cyanobacterial host on the one hand and diversification of cyanobacterial population on the other. In fact, Pollard and Young (2010) emphasized that the death of some cells in amongst *C. raciborskii* filaments reduces the filament size, thus enhancing the dispersal of both host and viruses by water currents.

A recent study conducted by Kimura *et al.* (2013) proposed a rapid coevolution between cyanobacteria and viruses resulting in diversification of both host (differing in viral resistance) and viruses (differing in host range). Such results place viruses as important biotic factor leading a cyanobacterial genotype reshuffle from year to year (Tanabe and Watanabe, 2011).

Whilst the profound influence exerted by viruses on cyanobacteria populations at short (population dynamics) and longer (evolutionary) timescales starts being well understood, a new frontier is to understand the source of spatio-temporal variations (e.g daily lysis rate of *Synechococcus* varies from 0.2- 46% in the Pacific Ocean while it did not exceed 1% in the Sargasso Sea (Matteson *et al.*, 2013) and 2% in the freshwater Lake Erie (Matteson *et al.*, 2011)) and to accurately model the impact of viruses on food web dynamics. Emerging systems biology approaches show great promise, in particular the combination of environmental metabolomics and transcriptomics (Vardi *et al.*, 2012; Rosenwassera *et al.*, 2014).

Allelopathy

Allelopathy, as defined here, applies only to the inhibitory effect of secondary metabolites produced by microorganisms on the growth, or physiology of cyanobacteria. Allelopathic compounds include alkaloids, cyclic and non-cyclic peptides, phenol, terpens and volatile organic compounds (Leão *et al.*, 2009). There is evidence showing that cyanobacteria could be affected by secondary metabolites released by diatoms (Leflaive and Ten-Hage, 2007), chlorophyceae (Gantar *et al.*, 2008) macrophytes (Hu and Hong, 2008) other cyanobacteria (Gross, 2003; Ahluwalia, 2013), and fungi (Mohamed *et al.*, 2014).

Sedmak *et al.,* (2008) tested the effect of planktopeptin BL (PP BL), anabaenopeptins (AnP) B and AnP F isolated from a *Planktothrix rubescens* bloom. These cyanobacterial products could completely suppress the growth of *Microcystis aeruginosa* even at very low concentration (10^{-7}-10^{-8} M) and induced cyanobacterial cell lysis. Additional investigations on the agent causing of cell lysis demonstrated that the PP and AnP were the triggers of a lysogenic virus to go to the lytic pathway. Nonetheless, allelopathic coumpounds produced by microorganisms can also present a strong direct impact on a target species. For instance, Mohamed *et al.,* (2014) showed that *Trichoderma citrinoviride*, a fungal species belonging to the phylum Ascomycota, could lyse *M. aeruginosa* cells and degrade the microcystin released by lysed cyanobacteria in few hours. Additionally, the authors reported that *T. citrinoviride* used both, the cells and the microcystin, for its growth.

Allelopathy also occurs among cyanobacteria. Rzymski *et al.* (2014) presented evidence that chemical products excreted by the cyanobacterium *Cylindrospermopsis raciborskii* inhibited the photosynthetic activity, decreased specific growth rate and induce cell necrosis resulting in a decrease of 36.2% of *M. aeruginosa* cells. The capacity of *C. raciborskii* to inhibit the PSII activity of several phytoplankton groups (chlorophyceae, diatoms and cyanobacteria) has been previously reported (Figueredo *et al.*, 2007). Authors of both studies hypothesised that allelopathy gives a strong competitive advantage to *C. raciborskii* and could be one reason for the expansion of this tropical cyanobacterium into temperate latitudes. Oberhaus *et al.* (2008) reported that *Planktothrix rubescens* TCC 29-1 excretes allelochemical compounds which drastically inhibits the growth of *P. agardhii* TCC 83-2 and to a lesser extent those of *P. agardhii* PMC 75.02 as well. This study demonstrated that allelopathy could occur between species of the same bloom-forming cyanobacterial genus.

While it has been shown that allelopathy may be behind unexplained variations in population dynamics in aquatic ecosystems (Legrand *et al.*, 2003; Ianora *et al.*, 2004), its role in the cyanobacterial bloom dynamic is still poorly studied. Such discrepancy could partially results from the challenge to lead investigations on secreted products in aquatic environments due to the dilution effect in the pelagic zone (Gross *et al.*, 2012).

Fungal Parasitism

Fungal infections are common in freshwater ecosystems (Van Donk and Ringelberg, 1983; Fernandez *et al.*, 2011; Rasconi *et al.*, 2012). In freshwater ecosystems, most of parasitic zoosporic true fungi belong to the polyphyletic Chytridiomycota (i.e. chytrids). This division is composed of approximately 1000 species previously grouped into five orders (Monoblepharidales, Chytridiales, Blastocladiales, Spizellomycetales, Neocallimastigales) distinguished on their mode of reproduction and spore ultrastructure (James *et al.*, 2006). However, the number of orders has been recently largely increased (Powell and Letcher, 2012). These fungi produce motile zoospores with a single posteriorly directed flagellum characteristic for the lineage, an adaption for dispersal in aquatic habitats (Shearer *et al.*, 2007). Furthermore, chytrids have been reported in soil (Freeman *et al.*, 2009), where a film surface water and capillary water allow zoospore dispersion (Gleason *et al.*, 2012). Whatever the species, the life cycle of all parasitic chytrid starts by the penetration of the host cell wall by rhizoids or infection pegs produced by the zoospores. A rhizoid system then develops, releasing extracellular

enzymes (proteases) that facilitate the digestion of cellular components, which are thereafter absorbed (Krarup *et al.*, 1994; Deacon, 2006). This fuels the development of the fungal reproductive structure, the sporangium, which consists of a sac-like structure undergoing multiple mitotic divisions resulting in a total conversion of its cytoplasm into zoospores (Berger *et al.*, 2005). After a maturation phase, zoospores are released into the environment. Gerphagnon *et al.,* (2013) give an illustration of each life stage reported for *Rhizosiphon crassum* and *R. akinetum*, two chytrid species which parasitize the cyanobacteria *Anabaena macrospora*.

Recently, several molecular environmental surveys (Lefèvre *et al.*, 2008; Le Calvez *et al.*, 2009) reported a high diversity of Chytridiomycota in aquatic habitats. Chytrid parasitism is common among eukaryotic algae in freshwater communities, but relatively few studies investigated the role of chytrid parasitism on cyanobacterial populations (Sigee *et al.*, 2007; Takano *et al.*, 2008). In 1972, Canter (1972) categorized fourteen chytrid species capable of infecting different genera of freshwater cyanobacteria. Some chytrids infect a wide range of hosts and sometimes even different species or genera. For example, *Rhizophydium megarrhizum* has been reported on *Aphanizomenon flos-aquae*, *Lyngbya sp*. and *Planktothrix agardhii* (Paterson, 1958; Davis *et al.*, 2003). In contrast, some species target specific host cells (i.e. akinetes or heterocysts) during cyanobacterial blooms (Canter, 1972; Takano *et al.*, 2008; Gerphagnon *et al.*, 2013). This specificity might possibly be ascribed to a possible chemotactic attraction between host and parasites (Mitchell and Deacon, 1986; Moss *et al.*, 2008). However, this hypothesis remains untested for phytoplankton parasites. Alternatively, Holfeld (2000) suggested that the host cell size could be one of the driving forces in chytrid infection by enhancing the encounter rate between zoospores and host cells. This hypothesis is supported as large and/or colonial phytoplankton species are more susceptible to chytrid parasitism (Ibelings *et al.*, 2004; Kagami *et al.*, 2007; Sime-Ngando, 2012).

Though chytrid parasitism on cyanobacteria was first noted as early as the late 19th century (Braun, 1856; De Wildeman, 1890) (Supplemental Table S3), its impact on cyanobacterial population dynamics remains largely poorly studied. However, it is likely to be similar to the well-established role of fungal parasitism in the decline of diatom blooms in freshwater ecosystems (Van Donk and Ringelberg, 1983; Sen, 1987; Bruning *et al.*, 1992; Holfeld, 2000). Previous studies with cyanobacteria have concentrated on taxonomic

identification, overlooking the trophic and functional interactions between the two organisms (Canter, 1951; Canter, 1954; Paterson, 1958; Canter, 1972). One of the first population studies investigated the dynamics of the host-parasite system *Anabaena planktonica* and *Rhizosiphon anabaenae* at Frain Lake, Washtenaw Country, Michigan, USA, for one year (Paterson, 1960). During this investigation, the increase of parasitism coincided with the decrease in the abundance of *A. planktonica* cells, suggesting that *Rhizosiphon anabaenae* contributed to the cyanobacterial bloom decline. However, as the author concluded, the collapse of the *A. planktonica* bloom may have been due to environmental physico-chemical factors rather than to fungal parasitism, considering the low level of infected cells (maximum prevalence 0.46%). In contrast, Canter (1951) discovered major parasitism of *Oscillatoria agardhii var. isothrix* (currently regarded as a taxonomic synonym of *Planktothrix isothrix*) by *Rhizophydium megarrhizum,* where 43% of host filaments were impacted by the chytrid and concluded that chytrid parasitism could influence the host health. This argument was supported by results obtained by Sen (1988) in research conducted over three successive years (from 1978 to 1980) in Shearwater, Wiltshire, United Kingdom. The author reported a maximum of 90% of cells of *Microcystis aeruginosa* parasitised by *Rhizidium microcystidis* and concluded that *R. microcystidis* played an important role in the decline of *M. aeruginosa* bloom. However, there were strong variations in the infection levels from year to year (80% in 1978, 15% in 1979 and 90% of cells that were parasitized in 1980). Similar variations were underlined by two field studies, conducted by Rasconi *et al.*, (2012) and Gerphagnon *et al.* (2013), in 2007 and 2010, respectively, in Lake Aydat, France, where maximum infection of *Anabaena macrospora* by *Rhizosiphon crassum* varied from 98% to 6% of vegetative cells. Finally, no relationship was found between the severity of infection and the recorded environmental parameters, suggesting that many interwoven factors and processes underpin these interannual variations.

Laboratory experiments could give some explanation of the influence of abiotic or biotic variables on chytrid infections. To our knowledge, just one study has examined fungal infection on cyanobacteria under laboratory conditions (Sonstebo and Rohrlack, 2011), probably due to the fact that the cultivation of chytrids requires considerable effort (Gleason *et al.*, 2007). In this work, Sonstebo and Rohrlack unveiled a close relationship between the chemotype (distinct cellular patterns of oligopeptides produced by different strains) of *Planktothrix rubescens* and *P. agardhii*, and the intensity of chytrid infection. In a recent study, Rohrlack *et al.* (2013) assumed that oligopeptides produced by cyanobacteria such as microcystins, anabaenopeptins, and microviridins could form important anti-parasite defenses against chytrids. Regarding the

heterogeneity of genotypes (Briand *et al.*, 2008) and chemotypes (Agha *et al.*, 2014) of cyanobacterial blooms under natural conditions, these results could provide an answer to the question of variability of the intensity of chytrid infection reported from year to year during field studies. Such inter annual variability of specificity between host and chytrids reinforces the Red Queen hypothesis (Bell, 1982), which predicts that organisms must constantly adapt to prevent predator or parasitic attacks.

The parasitism of cyanobacteria by eukaryotic organisms such as chytrids, forms an original system to investigate the evolution of "cross-kingdom host jump" in parasites (Van Baarlen *et al.*, 2007). However, the interactions within this pairing are still largely unknown. It is also crucial to increase the geographical scope of fungal parasitism studies as almost all reports of chytrid parasitism of cyanobacteria have been from studies in Europe, and U.S.A. with the exception of two preliminary Australian studies (Fabbro and Duivenvoorden, 1996; Gleason and Macarthur, 2008) and one Japanese (Takano *et al.*, 2008) study.

Lysis, Grazing and Parasitism of Cyanobacterial blooms: their interactions with the trophic web

During cyanobacterial blooms, photosynthetic prokaryotes can represent up to 100% of the total phytoplankton biomass (Bouvy *et al.*, 2001). Numerous studies have considered filamentous and colonial cyanobacteria as a trophic dead end (Porter and Orcutt, 1980; Fulton, 1988; Henning *et al.*, 1991; Jang *et al.*, 2003). However, bacterial and viral lysis, grazing and fungal parasitism via their involvement in the collapse of cyanobacterial blooms, may also pump the cyanobacterial matter and energy back into the ecosystem.

Bacterial or viral lysis of cyanobacterial blooms releases an important input of dissolved and particulate organic carbon in aquatic ecosystems. It is now well established that heterotrophic microorganisms, especially bacteria, play a major role in the carbon transfer from dissolved organic matter (DOM) from phytoplankton to metazooplankton, *via* flagellates and ciliates predation. This so-called microbial loop (or microbial food web when relationships with biogeochemical cycles are considered) (Azam *et al.*, 1983) has been described to be a major player of the established trophic web during a cyanobacterial bloom. Engström-Öst *et al.,* (2013) demonstrated that the trophic food web turned from autotrophic to heterotrophic after a

cyanobacterial collapse, which is explained by the fact that the lysis products of cyanobacteria could be used by bacteria and sustain all the bacterial production.

Food quality, rather than quantity, also regulates trophic transfer between primary producers and consumers (Müller-Navarra *et al.*, 2000). Usually, cyanobacteria are regarded as a poor food source for herbivorous zooplankton: they produce toxic substances (Chen *et al.,* 2005; Schwarzenberger *et al.,* 2010), lack essential nutrients (Von Elert *et al.,* 2003; Martin-Creuzburg *et al.,* 2008), and can form inedible colonies and filaments (Paerl and Otten, 2013; Rollwagen-Bollens *et al.,* 2013). Nonetheless, Perga *et al.* (2013) assumed that cyanobacteria could be considered as a complementary food for higher trophic levels. Additionally, the study led by Peduzzi *et al.,* (2014) underlined the importance of cyanobacteria for the trophic web in some ecosystems. The authors showed that a drastic suppression of cyanobacterial biomass caused by viruses could lead to a drastic decrease of birds in a bottom-up cascade. However, most of cyanobacteria released toxins when cells are lysed. Globally, these cyanotoxins are considered to have negative impacts on zooplankton communities (reviewed by Wilson *et al.,* 2006). Nevertheless, it has been shown that bacterial communities could rapidly degrade and use cyanotoxins for growth, especially when they are frequently exposed to toxins (Christoffersen *et al.*, 2002; Jones *et al.*, 2006). Toxins and lysis products could thus boost bacterial growth, resulting in the increase in the abundance of bacterivores such as ciliates, thereby stimulating the development of microzooplankton *via* the trophic cascade and the microbial food web (Christoffersen *et al.*, 1990; Engström-Öst *et al.*, 2013) (Fig.2). However, the accumulation of such cyanotoxins and their putative effects in the trophic web, especially in the microbial web, are still poorly understood. It was shown that flagellates can grow on cyanobacteria, which are toxic or not (Van Donk *et al.*, 2009; Wilken *et al.*, 2010). Furthermore, Bec *et al.,* (2006) demonstrated the importance of heterotrophic flagellates as a link between cyanobacteria and metazooplankton and indicated that heterotrophic flagellates can detoxify the toxic cyanobacteria *Microcystis aeruginosa* for the higher trophic level and therefore are responsible for trophic upgrading.

In the form of zoospores, chytrids can constitute up to 60% of the nanoheterotrophic flagellate fraction (0.6-5 µm) in autumn in freshwater lakes (Jobard *et al.*, 2010). Chytrid zoospores are efficiently grazed by daphnids and copepods (Kagami *et al.*, 2011) (Fig.2). They also produce sterols and polyunsaturated fatty acids (Masclaux *et al.*, 2011). The transfer of carbon from primary production to higher trophic levels *via* chytrid parasitism has been reported previously for a diatom-chytrid pairing (Kagami *et al,* 2007). The authors reported that

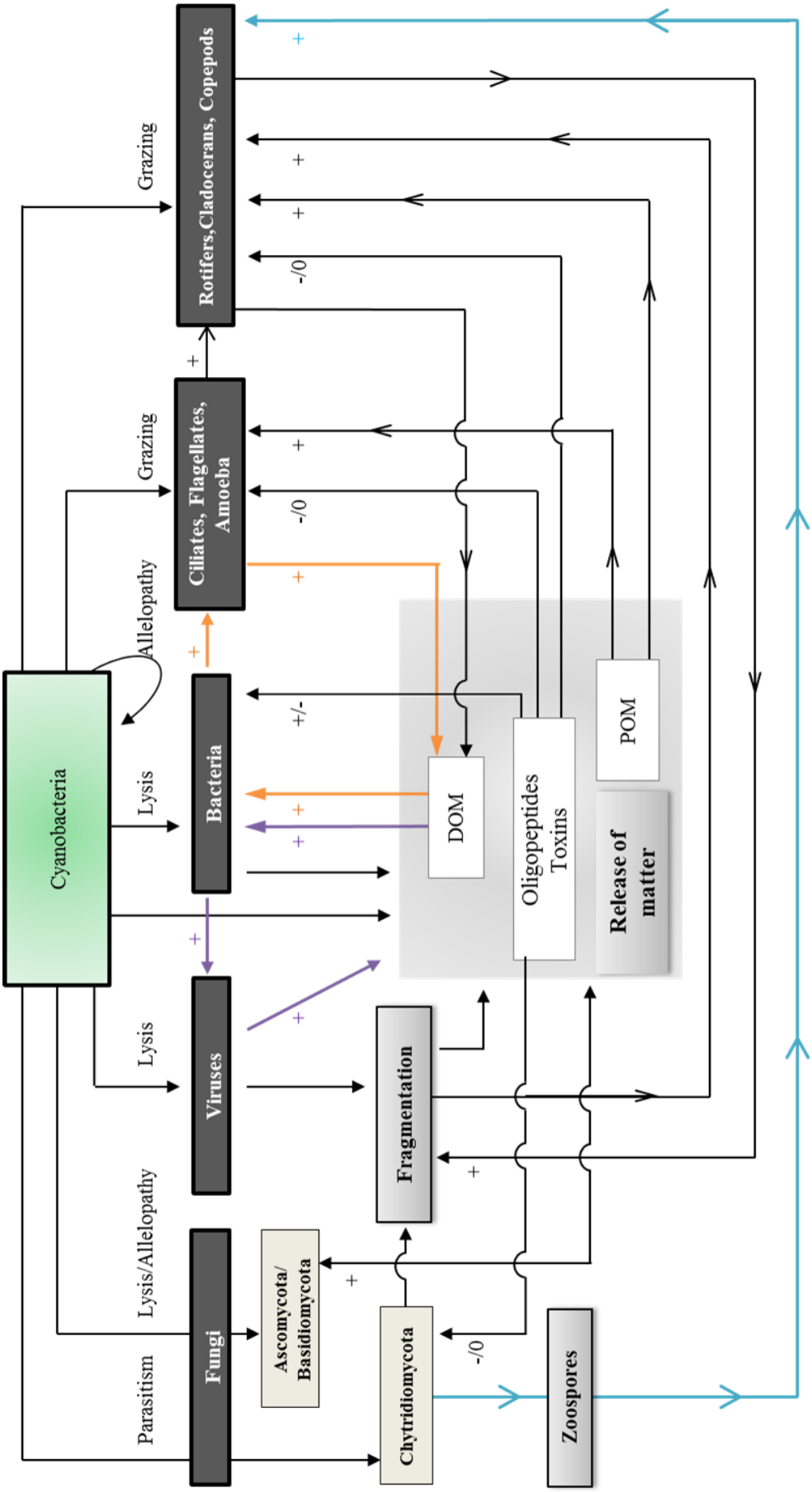

Figure 2: Conceptual model of impacts and interactions of five major microbial driving forces promoting the decline of cyanobacterial blooms.Trophic relations between microorganisms are represented by arrows with an highlight on loops and shunt, the viral shunt in purple, the microbial loop in orange and the mycoloop [previously defined by Kagami et al. (2014) as the trophic link established between inedible algae and zooplankton through the chytrid zoospores] in blue. +, positive effect; −, negative effect; 0, no effect.

fungi can upgrade the food quality of their host and constitute a link between inedible diatoms and higher trophic levels. In the same way, chytrids could be a real link between inedible cyanobacteria and metazooplankton. Considering that cyanobacteria are depleted of essential compounds, such as sterols and long-chain polyunsaturated fatty acids (Von Elert *et al.*, 2003) and that arthropods (cladocerans or copepods) are not capable of synthetizing *de novo* sterols and have to find them from their food, chytrid zoospores may be essential to transfer and upgrade biochemical food quality from filamentous cyanobacteria to metazoan community.

Chytrids can also reduce the length of cyanobacterial filaments by a so-called mechanistic fragmentation (Fig. 1D and Fig. 2), (Gerphagnon *et al.*, 2013) which could (i) promote the decline of the bloom especially for heterocystous cyanobacterial species (Chan *et al.*, 2004) (ii) enhance grazing (Bouvy *et al.*, 2001) and (iii) facilitate both host and parasite dispersal by water currents (Pollard and Young, 2010). However, both trophic upgrading and the mechanistic fragmentation due to chytrid parasites of cyanobacteria have to be experimentally tested in the general context of the effects of microbial parasites in food web dynamics.

In most studies, each biological factor involved in the decline of cyanobacterial blooms is treated independently, which fails to consider the complexity of aquatic ecosystems. Under natural conditions, several biotic factors, which have the same target host, co-exist. Manage *et al.,* (2001) reported the co-existence of the algicidal bacteria *Alcaligenes denitrificans* and cyanophages like particles, which participated simultaneously in the decline of *Microcystis aeruginosa* blooms. Unfortunately, the relative contribution of each lytic factor to the collapse has remained unclear. In previous studies, the quantification of the parts of viral lysis and of grazing involved in bacterial mortality has been done. Weinbauer and Höfle (1998) and Bettarel *et al.,* (2004) showed that two factors occurred in separate water layers in the eutrophic lake Plußsee, Germany and Lake Aydat, France, respectively. These two studies reported that in oxic water, the microbial loop dominated, as grazers removed the major part of bacterial production whereas in anoxic water, it was the viral loop. Both these two loops could enhance a depletion of carbon transfer efficiency to the metazooplankton by increasing the residence time of organic matter in the water column and favor bacterial respiration (Fuhrman, 1999). In the most recently described concept i.e. the mycoloop (Kagami *et al.*, 2014) such depletion of carbon transfer efficiency to higher trophic level may not be assumed. Actually, by taking their energy directly from living cells, chytrids avoid the bacterial step, which is indispensable in the microbial and viral loops. The nutrients from large inedible algae are transferred directly to

zooplankton *via* the zoospores of chytrids that have consumed nutrients from their large phytoplankton hosts. Therefore, compared to the two other loops, chytrid parasitism may improve the efficiency of carbon transfer to the grazers (Grami *et al.*, 2011; Rasconi *et al.*, 2014). Overall, it is essential to clearly quantify and to compare the efficiency of carbon transfer due to the three conceptual loops (i.e. from viruses, grazers and parasites), primarily during the decline of phytoplankton blooms.

Conclusion

It appears that the termination of cyanobacterial blooms is the result of a combination of several biotic and physicochemical factors (Christoffersen *et al.*, 1990; Kanoshina *et al.*, 2003; Zhang *et al.*, 2012). In contrast to these latter, the relative contribution of biotic factors as promoting agents in the decline of cyanobacterial blooms remains understudied. Experimentally, the difficulty lies in the fact that the impact of each biological factor seems to be closely dependent on the system under consideration, even for the most studied driving force, the grazers (Wilson *et al.*, 2006; Tillmanns *et al.*, 2008). It is clear that there is still no general conclusion regarding the capacity of grazers to control cyanobacterial blooms. However, it is clear that herbivores impact the structure (e.g size, strain composition) of cyanobacterial populations, depending on their respective specific characteristics. Additionally, host-parasite interactions are ecologically far more complex than predatory-prey relationships, where size ratios are important allometric predictors. It is clear that a unique biotic factor cannot be responsible for the total disappearance of cyanobacterial blooms, but it is the result of a set of trophic interactions and of the physicochemical environment as well. In addition, after being largely neglected in the trophic web, fungal parasitism could play a major role in both the decline of largely inedible cyanobacterial blooms and the transfer of nutrients to higher trophic levels.

We recommend that efforts should be maintained to clarify and quantify the effects of microbial parasites and their interactions with filamentous, colonial and other large size phytoplankton blooms in the studies of food web dynamics in the world aquatic systems. Moreover, due to increasing global temperatures and eutrophication, cyanobacterial blooms will continue to become more frequent world-wide (Elliott, 2011; Paerl and Otten, 2013). In parallel, some authors have emphasized that the global warming could affect phytoplankton-chytrids interactions (Ibelings *et al.*, 2011). The consideration of fungal infections on

cyanobacteria and modeling of cyanobacteria-chytrid parasitism evolution in a context of global change will be necessary to evaluate the real increase of cyanobacteria in aquatic ecosystems.

In aquatic environments, current experimental co-evolution studies remain mostly confined to prokaryotes and viruses (Brockhurst and Koskella, 2013). In the coming years, the combination of deep environmental sequencing (especially metatranscriptomics) with metabolomics, mesocosm and lab-based manipulation of fungal-cyanobacterial interactions will open new perspectives for the *in situ* characterisation of these pathogens contribution to food web fluxes.

Acknowledgements

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Supplemental material

Supplemental Table 1. Review of selected papers on grazer species of cyanobacteria

Cyanobacteria	Grazer species	Reference
Anabaena sp.	*Keratella cochlearis*	Gilbert (1990)
	Ceriodaphnia cornuta	Kâ *et al.,* (2012)
	Daphnia pulex,	Arnold (1971)
	D. longispina	Lampert (1987)
	Mesocyclops ogunnus	Kâ *et al.,* (2012)
	Moina micrura	Fulton (1988)
	Nassula sp.	Canter *et al.,* (1990)
	Pseudodiaptomus hessei	Kâ *et al.,* (2012)
Aphanizomenon sp.	*Daphnia pulex*	Holm *et al.,* (1983)
	Bosmina longispina maritima	Sellner *et al.,* (1994)
	Cyclopoid copepod	Yamamoto and Nakahara (2005)
	Daphnia pulicaria	Haney (1987)
Cylindrospermopsis sp.	*Daphnia magna*	Panosso and Lurling (2010)
	Brachionus calicyflorus	Soares *et al.,* (2010)
	Paramecium caudatum	Fabbro *et al.,* (2001)
	Unidentified copepod	Bouvy *et al.,* (2001)
Lyngbya sp.	*Asplanchna girodi*	Snell (1980)
	Diaptomus dorsalis	Havens and East (2006)
	Mesocyclops edax	Havens and East (2006)
Microcystis sp.	*unidentified amoebae*	Van Wichelen *et al.,* (2010)
	Daphnia magna	DeMott (1991)
	Daphnia pulicaria	Ghadouani *et al.,* (2004)
	Notodiaptomus iheringi	Panosso *et al.,* (2003)
Oscillatoria sp	*Diaptomus dorsalis*	Work and Havens (2003)
	Daphnia sp.	Haney and Trout (1982)
	Eudiaptomus graciloides	Haney and Trout (1982)
Planktothrix sp.	*Cyclops abyssorum*	Kurmayer and Juttner (1999)
	Daphnia hyalina	Kurmayer and Juttner (1999)
	Daphnia pulicaria	Oberhaus *et al.,* (2007)
	Nassula sp.	Combes *et al.,* (2013)

Supplemental Table 2. Review of selected papers on lysis entities of cyanobacteria

Cyanobacteria	Lysis or growth inhibitory agent		Reference
Anabaena sp.	Bacteria	*Bacillus cereus*	Zhao *et al.,* (2012)
	Bacteria	*Cytophaga sp.*	Rashidan and Bird (2001)
	Bacteria	*Pseudomonas sp.*	Kodani *et al.,* (2002)
	Virus	*Siphoviridae-A-CS1*	Deng and Hayes (2008)
	Virus	*Podoviridae/N(S)1*	Franche (1987)
	Virus	*Filamentous phage A-CF1*	Deng and Hayes (2008)
	Virus	*Myoviridae A-CM1*	Deng and Hayes (2008)
Aphanizomenon sp.	Bacteria	*Bacillus cereus*	Shunyu *et al.,* (2006)
	Virus	*Myoviridae-Ap-1*	Granhall and Vonhofst, (1969)
Cylindrospermopsis sp.	Bacteria	*Lysobacter cf. brunescens*	Flaherty *et al.,* (2007)
	Virus	*Siphoviridae-unidentified species*	Pollard and Young (2010)
Lyngbya sp.	Bacteria	*Lysobacter cf. brunescens*	Walker and Higginbotham (2000)
	Virus	*Siphoviridae-LPP-1*	Philips *et al.,* (1990)
Microcystis sp.	Bacteria	*Alcaligenes denitrificans*	Manage (2000)
	Bacteria	*Bdellovibrio-like bacteria*	Caiola and Pellegrini (1984)
	Bacteria	*Bacillus cereus*	Nakamura *et al.,* (2003)
	Bacteria	*Pseudomonas aeruginosa*	Zhang *et al.,* (2012)
	Virus	*Podoviridae M-CP1*	Deng and Hayes (2008)
	Virus	*Myoviridae-Ma-LMM01*	Yoshida *et al.,* (2006)
	Virus	*Filamentous phage M-CF1*	Deng and Hayes (2008)
	Cyanobacteria	*Cylindrospermopsis raciborskii*	Rzymski *et al.,* (2014)
	Cyanobacteria	*Planktothrix rubescens*	Sedmak *et al.,* (2008)
	Fungus	*Trichoderma citrinoviride*	Mohamed *et al.,* (2014)
Oscillatoria sp	Bacteria	*Pseudomonas sp.*	Kodani *et al.,* (2002)
	Bacteria	*Bacillus cereus*	Shunyu *et al.,* (2006)
Planktothrix sp.	Virus	*Myoviridae PZ1*	Deng and Hayes (2008)
	Virus	*Siphoviridae PZ10*	Deng and Hayes (2008)
	Virus	*Filamentous phage*	Deng and Hayes (2008)
	Virus	*unassigned PaV-LD*	Gao *et al.,* (2009)
	Cyanobacteria	*Planktothrix rubescens*	Oberhaus *et al.,* (2008)

Supplemental Table 3: Review of selected papers on parasitic chytrids of cyanobacteria

Cyanobacteria	Chytrid species	References
Anabaena sp.	*Rhizosiphon akinetum*	Canter (1954)
	Rhizosiphon anabaenae	Canter (1953)
	Rhizosiphon crassum	Canter (1953)
	Blastocladiella anabaenae	Canter and Willoughby (1964)
	Rhizophydium sp.	Gleason and Macarthur (2008)
	Rhizosiphon anabaenae	Canter (1972)
	Chytridium cornutum	Braun (1856)
	Blastocladiella anabaenae	Canter and Lund (1968)
	Rhizophydium sphaerocarpum	Canter (1972)
	Rhizosiphon crassum	Rasconi *et al*., (2012)
	Unidentified chytrids	Sigee *et al.*, (2007)
	Rhizosiphon crassum	Canter (1951); Gerphagnon *et al.,*(2013)
	Unidentified chytrid 1	Takano *et al.,* (2008)
	Unidentified chytrid 2	Takano *et al*., (2008)
	Rhizophydium ubiquetum	Canter and Lund (1968)
	Scherffeliomyces sp.	Canter (1972)
Aphanizomenon	*Blastocladiella anabaenae*	Canter and Lund (1968)
	Chytridium cornutum	Canter (1963)
	Phlyctidium globosum	Skuja (1956)
	Rhizophydium megarrhizum	Paterson (1958)
	Rhizophydium subangulosum	Fjerdingstad (1966)
Cylindrospermopsis sp.	*Unidentified chytrids*	Fabbro and Duivenvoorden (1996)
Lyngbya sp.	*Rhizophydium megarrhizum*	Paterson (1958)
Microcystis sp.	*Chytridium microcystidis*	Canter,(1972); Van Wichelen *et al.*, (2010)
	Rhizidium microcystidis	Canter (1972); Sen (1988)
	Rhizophydium sp.	Gleason and Macarthur (2008)
	Chytridium microcystidis	Canter (1972); Skuja (1948)
	Unidentified chytrid	Rasconi *et al.,* (2009)
	Unidentified chytrid	Paterson (1958)
Oscillatoria sp	*Rhizophydium subangulosum*	Fott (1950)
	Rhizophydium megarrhizum	Canter (1972)
	Rhizophydium deformans	Jaag and Nipkow (1951)
	Rhizophydium oscillatoriae-rubescentis	Jaag and Nipkow (1951)
	Rhizophydium sp.	Paterson (1958)
Planktothrix sp.	*Rhizophydium megarrhizum*	Davis *et al.,* (2003); Sønstebø and Rohrlack (2011)

Chapitre 2

Site d'étude & Méthodes

I. Présentation du site d'étude: le lac d'Aydat

Le lac d'Aydat est situé à environ 25km au Sud-Ouest de Clermont-Ferrand (Puy-de-Dôme), au Sud de la Chaîne des Puys (Fig.1). Situé à 837m d'altitude, il présente une superficie de 60.3 ha, une profondeur moyenne de 7m et maximale de 15m. L'éruption du Puy de la Vache et de Lassolas, il y a environ 8500 ans, a entrainé la formation d'une coulée basaltique ayant barré la vallée de la Veyre, donnant naissance au lac d'Aydat. Ainsi, ce lac est qualifié de lac naturel de barrage.

Figure 1 : Photographie aérienne du lac d'Aydat

L'alimentation en eau du lac est assurée à 75% par la rivière Veyre, le reste étant assuré par les précipitations et les cours d'eau temporaires s'écoulant sur l'ensemble des 30×10^6 m^2 de son bassin versant. Ce dernier est recouvert à 70% de prairies et pâturages, 15% de forêts, le reste se partageant entre territoires naturels (pelouse, broussailles) et terrains artificialisés, pour la plupart aux abords du lac. Ce lac est principalement dédié à l'activité piscicole et au tourisme estival.

Les différentes études menées sur le lac d'Aydat ont permis de révéler son caractère typiquement eutrophe. Il présente une zone hypolimnique anoxique d'Avril à Octobre et est assujetti à de récurrents blooms cyanobactériens estivaux. Par ailleurs, le lac d'Aydat est un lac dimictique, c'est-à-dire que l'ensemble de sa colonne d'eau se mélange deux fois par an, une première fois au printemps et une seconde à l'automne.

Afin de ne pas faire de répétitions avec les parties « matériel et méthodes » des études présentées dans ce manuscrit, les différentes stratégies d'échantillonnage ne seront présentées que dans ces dites études.

II. Méthodes utilisées

Afin d'étudier les dynamiques fines et les relations étroites reliant les cyanobactéries et leurs parasites fongiques j'ai utilisé le plus vieil outil employé en écologie microbienne, le microscope photonique.

1) Bref rappel historique de la microscopie

L'observation d'objets avec des lentilles se pratiquait déjà dans l'Antiquité. Cependant, il fallut attendre le XIIème siècle pour que la combinaison de plusieurs lentilles débouche sur l'invention du microscope. La microscopie (du grec *mikros*, petit, et *skopein*, examiner) prit son essor, sous l'impulsion d'un Français, Louis Joblot (1645-1723), Professeur Royal de Mathématiques à l'Académie Royale de Peinture et de Sculpture de Paris. Il consacra une grande partie de sa vie à développer des microscopes avec lesquels il étudiait de nombreux « animalcules ». Mais le plus connu des pionniers de la microscopie fut certainement le Hollandais Antoni van Leeuwenhoek (1632-1723) (Fig.2). Drapier d'origine, cet homme mit au point le microscope éponyme, microscope de van Leeuwenhoek, afin de contrôler la qualité des fils de draps et du tissage. Doté d'un plus fort grossissement que les précédents, ce microscope lui permit d'observer des organismes unicellulaires et des bactéries. Les progrès des lois fondamentales de l'optique entrainèrent une évolution considérable de la microscopie au cours du XVIIIème siècle. L'évolution de la médecine et de la biologie au cours du XIXème siècle accrurent le besoin de microscopies performantes. Ainsi, le microscope devint l'instrument principal de nombreuses disciplines et fut à l'origine du développement de l'écologie microbienne au cours du XIXème siècle.

Figure 2 : Antoni van Leeuwenhoek (1632-1723), et le microscope de van Leeuwenhoek qu'il inventa.

2) Un développement méthodologique nécessaire

L'étude des chytrides dans les environnements pélagiques d'eau douce s'est avérée complexe. En effet, les critères morphologiques permettant de distinguer un chytride (ex : système rhizoïdal, forme du sporange) d'autres organismes, tels que les choanoflagéllés ou d'autres groupes épiphytes des cellules phytoplanctoniques, ne sont que très difficilement observables en microscopie sans l'aide de coloration. Afin de faciliter l'étude de ces champignons aquatiques, une première méthode a été mise au point par Rasconi *et al.*, (2009). Cette méthode a fait l'objet d'un chapitre que nous avons publié dans Laboratory Protocols in Fungal Biology (2013), présenté ci-après. Brièvement, cette méthode consiste à colorer la paroi chitineuse des chytrides grâce à un fluorochrome, le CalcoFluorWhite (CFW). Grâce à cette coloration, la chitine émet une couleur bleue lorsqu'elle est excitée par des UV. Ainsi la paroi du sporange et le système rhizoïdal du chytride, deux critères morphologiques essentiels dans la détermination de ces champignons parasites, apparaissent distinctement (Fig. 3). Ceci permet alors une détermination plus aisée des chytrides. Cependant, cette méthode ne se limite qu'à l'étude du stade de vie parasitaire : le sporange. Au cours de ma thèse, j'ai donc développé une seconde méthode permettant d'étudier simultanément la phase parasitaire et la phase de dissémination des chytrides : les zoospores. Cette méthode est basée sur une combinaison de deux fluorochromes le CFW et le SYTOX-green. Le protocole sera présenté plus en détail dans la section suivante (b.).

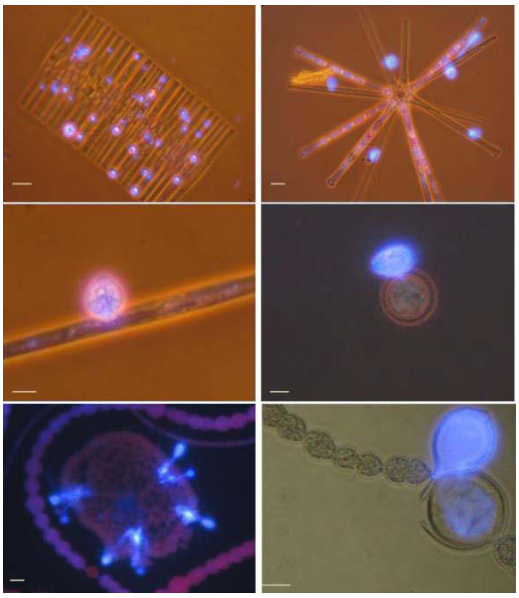

Figure 3: Application du fluorochrome Calcofluor white sur divers couple phytoplancton-chytride: A. *Fragilaria crotonensis-Chytridium* versatile and *Rhizophidium fragilariae* B. *Asterionella formosa-Rhizophidium planktonicum* C. *Synedra sp.-Rhizophidium planktonicum* D. *Cyclotella planctonica-Zygorhizidium sp*. E. *Woronichinia sp.- unidentified chytrid species* F. *Anabaena macrospora-Rhizosiphon akinetum.* Echelle=10µm

Les résultats écologiques révélés par cette méthode sont présentés dans le **Chapitre 4** de ce manuscript et a fait l'objet d'un article publié dans *Applied and Environmental Microbiology*.

a) Méthode basée sur le fluorochrome CalcofluorWhite

Article 2:

Diagnose of parasitic fungi in the plankton: technique for identifying and counting infective chytrids using epifluorescence microscopy

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Abstract

Fungal epidemics, especially in the form of parasitic chytrids, are omnipresent in aquatic environments, infecting diverse organisms. Major target hosts are algae, primarily diatoms, chlorophytes, and colonial or filamentous cyanobacteria. Chytrids are also called 'zoosporic' organisms because their life cycle includes dispersal forms, i.e. uniflagellate zoospores, and host-associated infective sporangia as well. They are considered relevant not only for the evolution of their hosts but also for the population dynamics and successions of phytoplankton communities, thus representing an important ecologically driving force in the food web dynamics. However, ecological knowledge of microscopic fungal parasites in aquatic environments is weak, compared to terrestrial ecosystems. We propose a routine protocol based on size fractionation of pelagic samples and the use of the fluorochrome calcofluor white (which binds to β-1,3 and β -1,4 polysaccharides) for diagnosing, identifying, and counting chitinous fungal parasites (i.e., the sporangia of chytrids). The protocol offers a valid method for the quantitative ecology of chytrid epidemics in aquatic ecosystems and food web dynamics.

Introduction

Fungal infections are recurrent in aquatic ecosystems (Sparrow, 1960, Rasconi*, et al.*, 2009). The most described aquatic fungi in freshwater ecosystems belong to Chytridyomycota (or chytrids). Chytrids infect a wide variety of hosts, including fish, eggs, zooplankton and other aquatic fungi but especially phytoplankton. Typical phytoplankton hosts include prokaryotes and eukaryotes, primarily large size diatoms and filamentous species (Lefèvre*, et al.*, 2006). Associated chytrids are external eucarpic parasites which produce specialized rhizoidal system within host cells, i.e. the diet conveying system that leads to the formation of the chitinous fruit bodies: the sporangium. This parasitic stage produces numerous uniflagellate spores: the zoospores, which constitute the dissemination phase of the life cycle (Lefèvre*, et al.*, 2008).

Various approaches have been used to study fungal parasites but routine techniques for reliably identifying and counting these organisms are missing in the context of aquatic microbial ecology (Sen, 1987, Kudoh & Tokahashi, 1990). So far, observations of parasitic fungi were obtained by using phase contrast light microscopy with live or Lugol's iodine preserved samples (Canter, 1951, Canter & Lund, 1951, Van Donk & Ringelberg, 1983). Such conventional microscopy allows observation of fungal sporangia or similar forms (especially in laboratory cultures), but is a poor approach for characterizing chytrid parasites in natural samples, at the complex community level. For example, a simple light microscopy observation of fungal rhizoidal systems, i.e. a pertinent criterium for identifying chytrids (Ingold, 1940, Huber-Pestalozzi, 1944, Lefèvre*, et al.*, 2008), is very difficult. This situation may help explain the confusion of chytrids with protistan flagellates such as choanoflagellates or other bacterivorous flagellates in the group of Bicosoeca, which are attached to phytoplankton but do not harm their host (Van Donk & Ringelberg, 1983, Kudoh & Tokahashi, 1990).

Earlier studies on chytrids were restricted to morphological descriptions and focused on few species (Canter, 1954, Canter & Lund, 1969, Beakes*, et al.*, 1988, Beakes*, et al.*, 1992). Electron microscopy was used to describe different life stages and the ultrastructural cytology of fungal zoospores and spore differentiation (Powell, 1978, Barr, 1992, Beakes*, et al.*, 1993), providing the basis for chytrid taxonomy (Cook, 1932, Reynolds, 1940). Studies on pelagic chytrids started in the British lakes (Canter & Lund, 1948), and different authors have provided descriptions of morphological characters (Canter, 1951, Pongratz, 1966, Canter, 1972, De Bruin*, et al.*, 2008, Gleason*, et al.*, 2008). However, few attempts have been made to include the related parasitism pathway in the aquatic food web dynamics, and to understand

environmental factors that trigger epidemics as well (Rasconi, *et al.,* 2011*).* Some authors have also investigated the effects of parasitism on the growth of algal host species and on the genetic structure of infected populations. Parasites are thus considered relevant not only for the evolution of their hosts but also for the population dynamics such as successions of phytoplankton communities, and for structuring microbial communities in general (Van Donk, 1989). Moreover, chytrids can represent interesting key intermediates in the food chain (Kagami, et al., 2007, Gleason, et al., 2009, Masclaux, et al., 2011). The nutrients from infected large size algae which could not be directly fed by zooplankton can be transferred from sporangium to grazers via fungal zoospore production. Fungal zoospores have suitable dimensions and represent a valuable food source for zooplankton *(Kagami, et al., 2007). The* activity of zoosporic fungi and the related biogeochemical processes can thus be crucial in matter and energy transfer in aquatic systems (Rasconi*, et al.*, 2011). Methodological limitations for the study of the ecological dynamics of chytrid populations can be overcome with epifluorescence microscopy coupled to a specific fluorochrome targeting molecular tracers (i.e. some types of polysaccharides) of the fungal chitinous structures, including sporangium and the rhizoidal system. The protein stain fluorescein isothiocyanate (FITC) and, in particular, the chitin stain calcolfluor white (CFW), were suggested as good markers that offer useful tools for the investigation of fungal dynamics in aquatic samples (Müller & Sengbusch, 1983). CFW binds to β1-3 and β1-4 polysaccharides such as those found in cellulose or in chitin which commonly occur in the fungal cell wall (Rasconi*, et al.*, 2009). It fluoresces when exposed to UV light and is currently used in clinical mycology for direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimen for fungal elements (Harrington & Hageage, 2003). In contrast to FITC, CFW penetrates into infected host cells and is more efficient for the observation of the complete rhizoidal system of parasites, i.e. a pertinent criterion for chytrid identification (Canter, 1951, Sparrow, 1960).

The main objective of this Chapter is to provide, in a simplified step-by-step format, a routine protocol based on size-fractionation of pelagic samples and the use of the fluorochrome calcofluor white for diagnosing, identifying and counting chitinous fungal parasites (i.e. sporangia of chytrids) within phytoplanktonic communities (Rasconi*, et al.*, 2009), together with practical advices on how to apply the method.

Materials

1. 25 µm nylon filter
2. 0.2 µm filters
3. High performance concentration /diafiltration system. As an example, we use the system Amicon model DC 10LA (Epernon, France) equipped with a reusable hollow fiber cartridge (0.2 µm cutoff, surface area of 0.45 m^2)
4. 36% Formaldehyde
5. Calcofluor white ($C_{40}H_{44}N_{12}O_{10}S_2$ Fluorescent Brightener 28; Sigma catalog no. F3543)
6. 10N NaOH
7. Balance
8. Distilled water
9. 15 ml and 0.2 ml tubes
10. Glass slides and cover slips
11. Epifluorescence microscope equipped with appropriate UV filter sets and Neofluar objective lens (optional).

Methods

Concentrations of cells (*see Note 1*)

1. Pass the sample (ca. 20 L) trough the 25 µm pore size nylon filter (*see Note 2*).
2. Collect large phytoplankton cells in the > 25 µm size fraction by washing the filter with 40 mL of 0.2 µm filtered lake water
3. Fix the concentrate sample with formaldehyde (2% final conc.), before staining and analysis.
4. Concentrate nanoplanktonic cells in the < 25 µm size-fraction (i.e. the 20 L filtrate) ca 20x by ultrafiltration to a volume of approximately 1 L, entry pressure 0.9 bar.
5. Fix about 180 mL of the ultrafiltrate retentate with formaldehyde (2% final conc.), before staining and analysis

Preparation of Calcofluor stock solution

1. Weigh 35 mg of Calcofluor White into a 15 mL tube.
2. Add 7 mL of sterile distilled water and 2-3 drops of 10N NaOH (to increase pH to 10-11). Calcofluor does not dissolve well in neutral solutions.

3. Dissolve the calcofluor
4. Adjust the volume to 10 mL by adding sterile distilled water.
5. Distrubute the stock solution in 0.2 ml tubes and store in a lightproof tube at -20°C.

Staining and visualization

6. In the dark, stain aliquots (about 200 µl) of concentrated and fixed materials by adding 1 to 2.5% (vol/vol) of CFW stock solution directly in solution for 10 minutes.
7. Mount drop (5-10 µl) of the stained samples between glass slides and cover slips for observations and counting.
8. In a dark room, examine the slides under an epifluorescence microscope equipped with appropriate set of filters and objective lens. Shift between white and UV light to visualize and determine parasites and phytoplankton cells, and check the viability of the host cell, e.g. presence of chloroplast.
9. Applied a standard procedure for microscopic counting (*see Notes 3 and 4*)

Notes

1. Different approaches were tested to concentrate samples: the total community approach and the size-fractionated community approach. For the former approach, 180 ml of experimental samples were fixed with formaldehyde (2% final conc.) and aliquots concentrated in three different ways: (i) by simple gravity following Utermöhl's method (Utermöhl, 1958) before staining the chyrids, (ii) by vacuum pressure on two different filters before staining directly onto filters, and (iii) by vacuum pressure on the same two types of filters but after staining in solution.

For the Utermöhl method, 100 ml of fixed samples were settled for at least 24 h. For each of the two filter-vacuum pressure methods, 10 ml x 2 of fixed samples were filtered onto polycarbonate white filters (pore size 0.6 µm, catalog no. DTTP02500, Millipore) and nuclepore polycarbonate black filters (pore size 0.8µm, catalog no. 110659, Whatman), by using gentle vacuum (< 0.2 bar or 20 kPa). For the total community approach using the classical Utermöhl method, visualization of fungal parasites was very difficult and most of the time practically impossible for all the stain concentrations tested. The main reason was that staining directly in the Utermöhl chamber resulted in very poor-quality specimens of parasites observed

in any given sample. Other disadvantages of the procedure include the relatively long sedimentation time and the difficulty of increasing the volume analysed.

The alternative total community approaches based on vacuum pressure concentrations on polycarbonate filters, i.e. white (0.6-µm-pore-size) and black (0.8-µm-pore-size) filters, yielded similar quality images of fungal parasites, either when CFW staining was done before (i.e. in solution) or after (i.e. on filters) concentrating phytoplankton host cells onto filters. However, substantial differences were noted depending both on the type of the filter and on the concentration of the stain. In general, for the two types of filters, high levels of background noises were obtained when using CFW at final concentrations of 3, 10 or 20%, precluding any accurate assessment of numerical and phenotypic characteristics of both host cells and their fungal parasites. Staining with 1% CFW final concentration substantially improved the viewing of chytrids on filters, with an increasing contrast from the white DTTP Millipore to the black Whatman filters. However, none of the membrane-retaining approaches yielded satisfactory images of morphological and cellular features of the host cells, e.g. presence of chloroplast, viability of the host cell. Accordingly, the propose protocol is based on the size-fractionation approach using 1 to 2.5% vol/vol CFW final concentration (from the stock solution), which substantially enhanced the observational results.

2. The approach is efficient since it is based on the concentration of large initial volumes and size-partitioning of samples, a step that we judged necessary in order to yield good analytic images of infectious sporangia for an accurate diagnosing and identification of parasites. In addition, this approach yielded satisfactory images of morphological and cellular features of the host cells, for phytoplankton identification based on phenotypic features and viability of the host cell, through the integrity of cell wall and the presence of chloroplasts, which are fundamental parameters to assess the intensity of the disease. We consider this protocol as an optimal for the diagnosis and quantitative assessment of phytoplanktonic chytrid infections in natural samples. Finally, the approach was designed to freeze-conserved particulate DNA samples for quantifying the propagule stages (i.e. zoospores) of chytrids via FISH targeting of specific rRNA oligonucleotide probes.

3. To estimate the infectivity parameters of ecological interest in phytoplankton population, several algorithms are used according to formula proposed by Bush *et al*., (1997). These parameters include the prevalence of infection (Pr), i.e. the proportion of individuals in a given phytoplankton population having one or more sporangia or rhizoids, expressed as Pr (%) = [(Ni

/ N) x 100], where Ni is the number of infected host cells and N the total number of host cells. The second parameter is the mean intensity of infection (I), calculated as I = Np / Ni where Np is the number of parasites and Ni is the number of the infected individuals within a host population.

4. We propose a third parameter concerning the prevalence of infection of cells in colonial (or filamentous) species (Pr_{CF}). Pr_{CF} (%) = [(Ni / N) x 100] where Ni is the number of infected host cells in parasitized colonies (or filaments) and N the total number of parasitized host colonies (or filaments).

Acknowledgements

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b) Méthode de co-marquage mettant en jeu deux fluorochromes : le CalcoFluorWhite et le SYTOX-green

La méthode précédemment décrite, basée sur le CFW est un bon outil pour la détermination des chytrides. Cependant, le CFW se lie spécifiquement à la chitine et ne permet donc pas la visualisation des zoospores. En effet, ces spores flagellées sont dépourvues de paroi chitineuse. Cependant, au cours de cette thèse nous avons voulu nous intéresser aux relations existantes entre l'hôte et le parasite chytridien. Plus précisément, nous avons voulu mettre en évidence les différents facteurs influençant la fécondité[5] du parasite. Afin de répondre à nos interrogations, il nous a fallu développer une méthode nous permettant de visualiser les zoospores produites au sein de chaque sporange tout en gardant l'accessibilité, à l'identification de l'hôte et à l'identification du chytride, ainsi qu'aux paramètres classiques d'infection fongique, à savoir la prévalence d'infection (% de cellules hôtes infectée au sein d'une population donnée) et l'intensité d'infection (nombre moyen de sporanges par cellule infectée). Pour ce faire, nous avons mis au point une méthode de co-marquage combinant le CFW avec

[5] Fécondité d'un chytride : Capacité de production des zoospores

le SYTOX-green. La description de la mise au point de cette méthode ainsi que les résultats écologiques obtenus sont présentés et discutés dans le **Chapitre IV.**

Comme précédemment, le CFW nous permettait de déterminer l'espèce fongique rencontrée et de mesurer les paramètres classiques d'infection fongique. Grâce à la spécificité du SYTOX-green envers les acides nucléiques, nous avons pu déterminer le nombre de zoospores produites au sein de chaque sporange mature, en illuminant le noyau de chaque spore. Puisque ces deux colorants ne sont ni excités par les mêmes longueurs d'onde, ni n'émettent dans le même spectre lumineux, leur association s'est avérée un bon outil pour l'analyse des relations fines existant entre les deux partenaires de la relation parasitaire. Les conditions optimales d'application de notre méthode de co-marquage sont données ci-dessous :

- L'échantillon utilisé est issu du fractionnement de la communauté phytoplanctonique. Cette dernière est concentrée sur 25µm, puis fixée au formaldéhyde (2% final). C'est sur un aliquot de ce concentrat que la méthode est alors appliquée.
- Le CFW est utilisé à une concentration finale de 2.5% vol/vol (de la solution mère).
- Le SYTOX-Green est utilisé à une concentration finale de 0.1µM.
- L'anti-fadding utilisé est préparé comme suit : 800µl de Citifluor et 200µl de VectaShield (Vector Laboratories)
- Incuber, au noir et à température ambiante l'échantillon (196µl de concentrat), et 4µl de SYTOX-green à 5µM pendant 40min.
- Ajouter le CFW (2µl de solution mère) et laisser incuber 5min au noir et à température ambiante
- Monter entre lame et lamelle 5 à 10µl de l'échantillon auquel 5µl d'anti-fadding ont préalablement été rajoutés.
- L'observation de la lame se fait grâce à un microscope à épifluorescence. La lumière UV (405 nm) excite le CFW qui apparait bleu, le SYTOX-green quant à lui apparait vert lorsqu'il est excité par une lumière bleue (488nm).

Cette méthode a été utilisée dans les **Articles 4 et 5** présentés respectivement dans les **Chapitres III et IV**. Au cours de ces deux études, nous nous sommes focalisés sur une des deux espèces de chytrides infectant la cyanobactérie *Anabaena macrospora* : *Rhizosiphon akinetum*. Ainsi, nous avons pu déterminer un facteur de conversion (CF). Une fois déterminé,

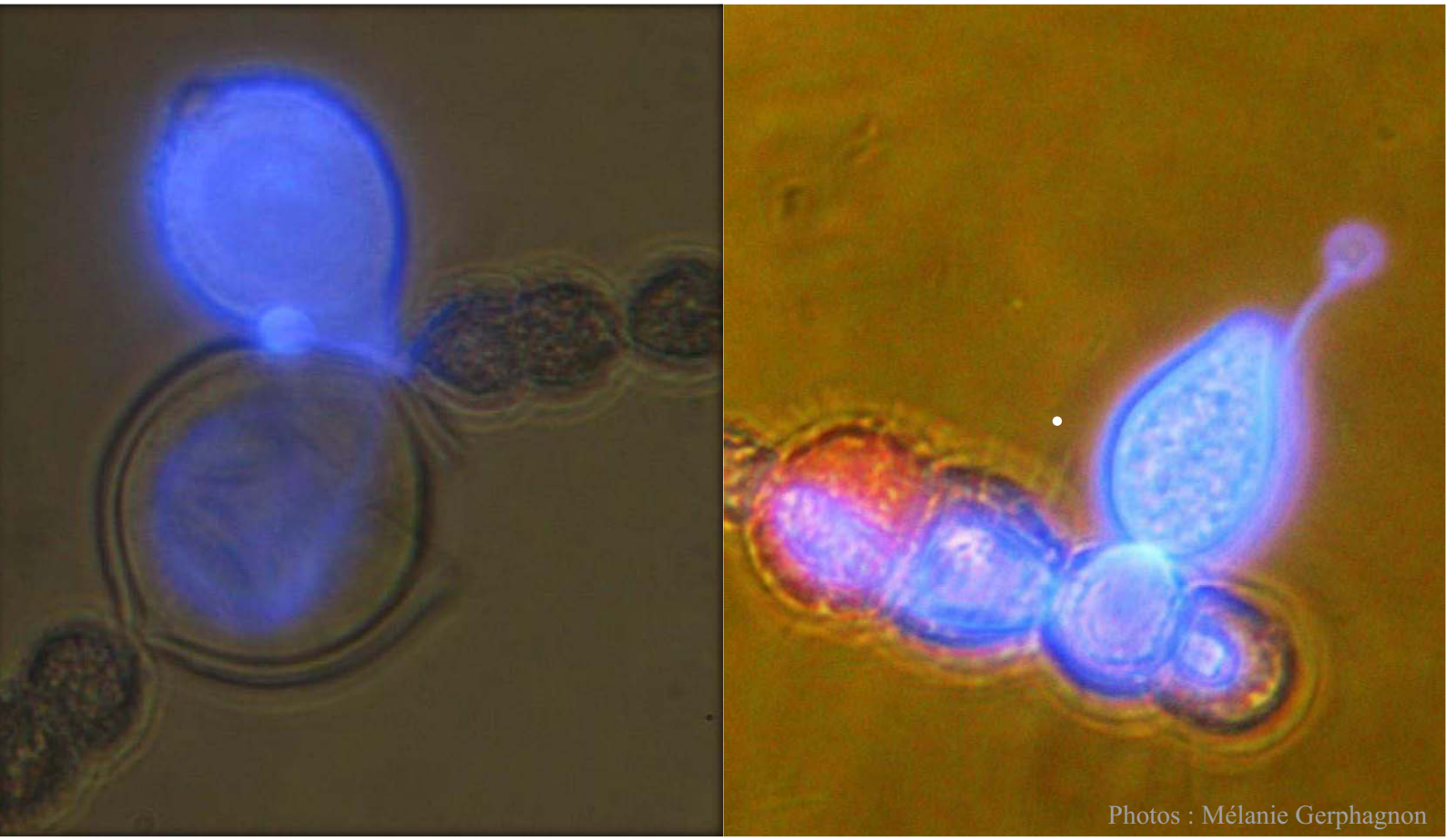
ce facteur de conversion permet d'accéder au nombre théorique de zoospores produites par un sporange en ne mesurant que son biovolume.

$$\mathrm{CF(nbr\ de\ zoospores.\ \mu m3\ de\ sporange)} = \frac{\mathrm{Nz\ (Nombre\ de\ zoospores\ dans\ un\ sporange\ mature\ (S))}}{\mathrm{VM.A\ (Volume\ du\ sporange\ (S)\ en\ \mu m3)}}$$

Par ailleurs, en rapportant le nombre de zoospores par μm^3 de sporange, ce facteur de conversion (CF) permet de comparer la capacité de fécondité de différentes espèces (Gerphagnon*, et al.*, 2013). Aussi, ai-je appliqué cette méthode et déterminé le CF d'une seconde espèce fongique parasitant *Anabaena macrospora* et rencontrée majoritaire lors de la première année de suivi : *Rhizosiphon crassum* (les résultats de cette étude sont présentés et discutés dans les **résultats complémentaires** du **Chapitre IV** de ce manuscrit). De la même manière le CF de *Rhizophidium fragilariae*, chytride parasitant la diatomée *Fragilaria crotonensis*, a été déterminé (les résultats sont présentés et discutés dans l'**Article 5** de ce manuscrit).

Chapitre 3

Anabaena macrospora-Rhizosiphon spp. : Étude de la dynamique spatio-temporelle de couples hôte-parasite



Photos : Mélanie Gerphagnon

Préambule

L'analyse bibliographique a révélé que les études précédemment menées sur les relations parasitaires existantes entre les chytrides et le phytoplancton se sont principalement intéressées à l'impact du parasitisme fongique sur les cellules phytoplanctoniques eucaryotes. En effet, bien que l'existence d'un tel parasitisme chez les cyanobactéries ait été reportée ((Canter, 1972) ; Cf synthèse bibliographique) les investigations menées sur les relations hôte-parasite mettant en jeu un hôte cyanobactérien et un parasite fongique sont relativement rares et éparses (Paterson, 1960, Sen, 1988, Takano*, et al.*, 2008). Dans un contexte de forçage anthropique extrêmement important sur les écosystèmes lacustres ayant conduit à l'augmentation des « blooms » cyanobactériens, il nous paraît essentiel de mieux appréhender l'impact du parasitisme fongique sur la dynamique de « blooms » cyanobactériens. Les premières études des communautés fongiques menées sur le lac d'Aydat ont permis de mettre en évidence la dynamique annuelle du parasitisme fongique associée à la communauté phytoplanctonique (Rasconi*, et al.*, 2009, Rasconi*, et al.*, 2012). Ces études ont ainsi pu mettre en évidence des infections fongiques non négligeables associées aux cyanobactéries, et plus particulièrement à la cyanobactérie filamenteuse *Anabaena macrospora*.

Dans ce chapitre seront présentées deux études dont la première a pour objectifs (i) de préciser les dynamiques hôte-parasite lors d'un bloom cyanobactérien, (ii) de déterminer l'impact du parasitisme fongique sur le déclin de ce bloom, et (iii) de décrire les cycles de vie des chytrides associés à ce bloom (**Article 3**). La seconde étude a pour objectif principal l'analyse de la répartition spatiale, tant horizontale que verticale, de paramètres fonctionnels caractérisant l'infection fongique (prévalence et intensité d'infection, et production de zoospores) associée aux efflorescences de la cyanobactérie *Anabaena macrospora* (**Article 4**).

Article 3 :

Fungal parasitism: life cycle, dynamics and impact on cyanobacterial blooms

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Abstract

Many species of phytoplankton are susceptible to parasitism by fungi from the phylum Chytridiomycota (i.e. chytrids). However, few studies have reported the effects of fungal parasites on filamentous cyanobacterial blooms. To investigate the missing components of bloom ecosystems, we examined an entire field bloom of the cyanobacterium *Anabaena macrospora* for evidence of chytrid infection in a productive freshwater lake, using a high resolution sampling strategy. *A. macrospora* was infected by two species of the genus *Rhizosiphon* which have similar life cycles but differed in their infective regimes depending on the cellular niches offered by their host. *R. crassum* infected both vegetative cells and akinetes while *R. akinetum* infected only akinetes. A tentative reconstruction of the developmental stages suggested that the life cycle of *R. crassum* was completed in about 3 days. The infection affected 6% of total cells (and 4% of akinètes), spread over a maximum of 17% of the filaments of cyanobacteria, in which 60% of the cells could be parasitized. Furthermore, chytrids may reduce the length of filaments of *Anabaena macrospora* significantly by "mechanistic fragmentation" following infection. All these results suggest that chytrid parasitism is one of the driving factors involved in the decline of a cyanobacteria blooms, by direct mortality of parasitized cells and indirectly by the mechanistic fragmentation, which could weaken the resistance of *A. macrospora* to grazing.

Introduction

Most parasitic zoosporic true fungi found in lakes belong to the phylum Chytridyomycota (i.e. chytrids) (Jobard, *et al.*, 2010). Although significant progress has recently been made with new approaches designed to assess their diversity and potential functions (Rasconi, *et al.*, 2009, Jobard, *et al.*, 2010, Lefèvre, *et al.*, 2010, Kagami, *et al.*, 2011, Monchy, *et al.*, 2011, Sonstebo & Rohrlack, 2011), these fungi are still poorly studied in pelagic systems. Furthermore, little is known about their dynamics in freshwater food webs. Large and/or colonial phytoplankton species are particularly susceptible to chytrid parasitism (Ibelings, *et al.*, 2004, Kagami, *et al.*, 2007), and are known to transfer matter and energy to zooplankton *via* grazing of fungal zoospores, through the so-called 'mycoloop' (Kagami, *et al.*, 2007). The most frequently studied models of fungal parasites of phytoplankton are those which infect eukaryotic hosts, primarily diatoms and chlorophytes, and declines in the size of blooms can be accelerated by parasitism (Canter & Jaworski, 1979, Beakes, *et al.*, 1988, Bruning, 1991, Shin, *et al.*, 2001, Kagami, *et al.*, 2007, Grami, *et al.*, 2011).

Most of the studies on the causes of filamentous cyanobacterial bloom decline have primarily considered physico-chemical factors, such as temperature, light, and the availability of nutrients (Robarts & Zohary, 1987, De Nobel, *et al.*, 1998, Downing, *et al.*, 2001, Herrero, *et al.*, 2001, Burford & Davis, 2011). Predation has been studied, but it has a weak impact on population densities of colonial or filamentous cyanobacteria (Lampert, 1987, Gliwicz, 1990, DeMott, *et al.*, 2001). Several studies have also investigated the impact of viruses on marine and freshwater cyanobacterial populations, clearly highlighting the effects of viruses on cyanobacterial bloom collapse (Hennes, *et al.*, 1995, Sigee, *et al.*, 1999, Weinbauer & Rassoulzadegan, 2004, Baker, *et al.*, 2006, Honjo, *et al.*, 2006, Pollard & Young, 2010).

Despite its potential roles in the decline of filamentous cyanobacteria, chytrid parasitism remains poorly studied. The few published studies on cyanobacterial-chytrid interactions were based on laboratory experiments with a focus on taxonomic description of fungi and host-parasite interactions, or on field studies, the durations of which largely exceed the generation times of both hosts and parasites (Canter, 1972, Sen, 1988, Takano, *et al.*, 2008, Sonstebo & Rohrlack, 2011). To overcome the bias of cultivation conditions, and their putative alteration of host-parasite interactions (De Bruin, *et al.*, 2008), and to provide an accurate host-parasite couple dynamic, we surveyed a recurrent chytrid-cyanobacterium assemblage in a productive lake using a fine resolution sampling strategy over the entire

bloom period of the filamentous heterocystous cyanobacterium *Anabaena macrospora.* Our specific objectives were to (i) analyze the dynamics of parasites and describe the different stages of their life cycles using direct microscopic observations, (ii) determine the infective strategies of chytrids in the different cellular niches (i.e. akinetes, vegetative cells) offered by the host, and (iii) infer the putative role of fungal parasitism in the decline of cyanobacterial blooms.

Materials and methods

Study site and sample collection

Samples were collected in Lake Aydat (45°39'48''N, 002°59'04''E), a small eutrophic lake (Zmax = 15 m, surface area = 60 ha) with a large catchment area (3 x 10^4 ha) located in the French Massif Central, where recurrent blooms of cyanobacteria occur in late summer and early autumn. Samples were collected every 3 days from the 6th of September to the 30th of October 2010, corresponding to the seasonal bloom of *Anabaena macrospora*. Samples were taken from the center of the lake at the point of maximum depth. To comprehend the vertical distribution of cyanobacteria and associated chytrid parasites, samples were collected at two different depths during each sampling date. The first was at a constant discrete subsurface depth (0.5m) and the second varied from 1 to 4m and depended on the maximum depth of fluorescence (MF) determined *in situ* from the vertical pigment profiles obtained by a BBE Fluoroprobe® (Moldaenke, Germany). 20 liters were sampled using an 8-L Van Dorn bottle. To eliminate the metazoan zooplankton, collected samples were immediately prefiltered through a 150 µm-pore-size nylon filter, poured into clean transparent recipients, and then transferred immediately to the laboratory for processing. The fraction >150µm was verified to ensure that no cyanobacterial cells were retained.

No specific permits were required for the described field studies, as the location is not privately-owned or protected in any way, and the field studies did not involve endangered or protected species

Physico-chemical parameters.

Water transparency was measured *in situ* with a Secchi-disk. Temperature and dissolved oxygen profiles were obtained using a multiparameter probe ProOdO^{TM} (Ysi, Germany). For determination of nitrate, ammonium and orthophosphate, 3 replicates × 50 ml of sampled

waters were filtrated through 0.2µm syringe filter and stored frozen at -20°C until analysis using spectroquant reagent standard kits (Merck, Germany).

Phytoplankton community

For phytoplankton analyzes, triplicate 180 ml of raw samples were fixed with Lugol's iodine and 5 to 20 ml subsamples (for each replicate and depending on the phytoplankton density) were settled overnight in counting chamber and cells counted under an epifluorescence microscope (Zeiss Axiovert 200M) according to the classic Utermöhl method. At least 400 cells were counted on at least 30 randomly selected optical fields. Phytoplanktonic cells were identified, at times up to the species level, using morphological taxonomic keys known from references books (e.g., (Geitler, 1932, Bourrelly, 1966)). The targeted cyanobacterium *A. macrospora* [misidentified as *Anabaena flos aquae* in Rasconi *et al.* (2012)], was quantitatively analyzed. The two species were distinguished on the basis of trichomes and the form and size of akinetes. *A. macroscopora* exhibits a straight single trichome and rounded akinetes (size 16-18µm wide, 17-26µm long) while *A. flos aquae* has a coiled trichome that forms a solitary or more usually an entangled twisted mass, with cylindrical or slightly ellipsoid and often slightly bent akinetes (size 6-13µm wide, 20-50µm long) (Whitton*, et al.*, 2002). The total numbers of *Anabaena macrospora* cells (vegetative cells and akinetes) and filaments in the samples were recorded and inspected for chytrid infection.

Chytrid parasitism

For chytrid infection parameters, samples were treated using the size-fractionated community method developed by Rasconi *et al*. (2009). Briefly, 18L of sampled water was concentrated on 25µm pore size nylon filter. Large phytoplankton cells (≥ 25µm), including the filamentous cyanobacteria *A. macrospora*, were collected by washing the filter with 0.2µm-pore-size-filtered lake water, fixed with formaldehyde (2% final concentration), and an aliquot of 195µl was stained for the chitin cell wall characteristic of fungi. We used the fluorochrome calcofluor white (CFW, $C_{40}H_{44}N_{12}O_{10}S_2$, excitation wavelength 440nm; emission wavelength 500-520nm, Sigma catalog no F3543) at a concentration of 2.5% (vol/vol), from a stock solution (Rasconi*, et al.*, 2009). Staining lasted at least 10 minutes before mounting between glass slides and cover slips and observation under the epifluorescence microscope (Zeiss Axiovert 200M) using UV light excitation at ×400 magnification. We systematically inspected 50 filaments comprising 780 to 2085 individual cells of *A. macrospora* to determine (i) the number of infected and non-infected cells and

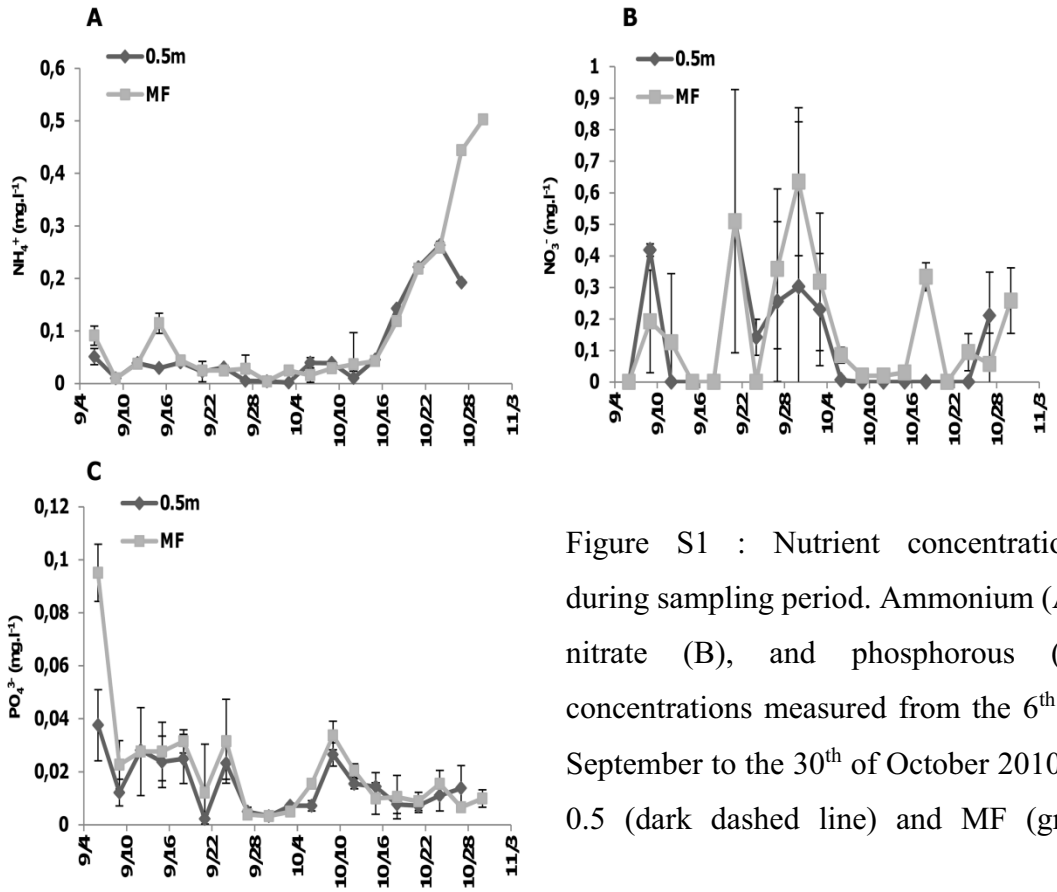

Figure S1 : Nutrient concentrations during sampling period. Ammonium (A), nitrate (B), and phosphorous (C) concentrations measured from the 6th of September to the 30th of October 2010 at 0.5 (dark dashed line) and MF (grey

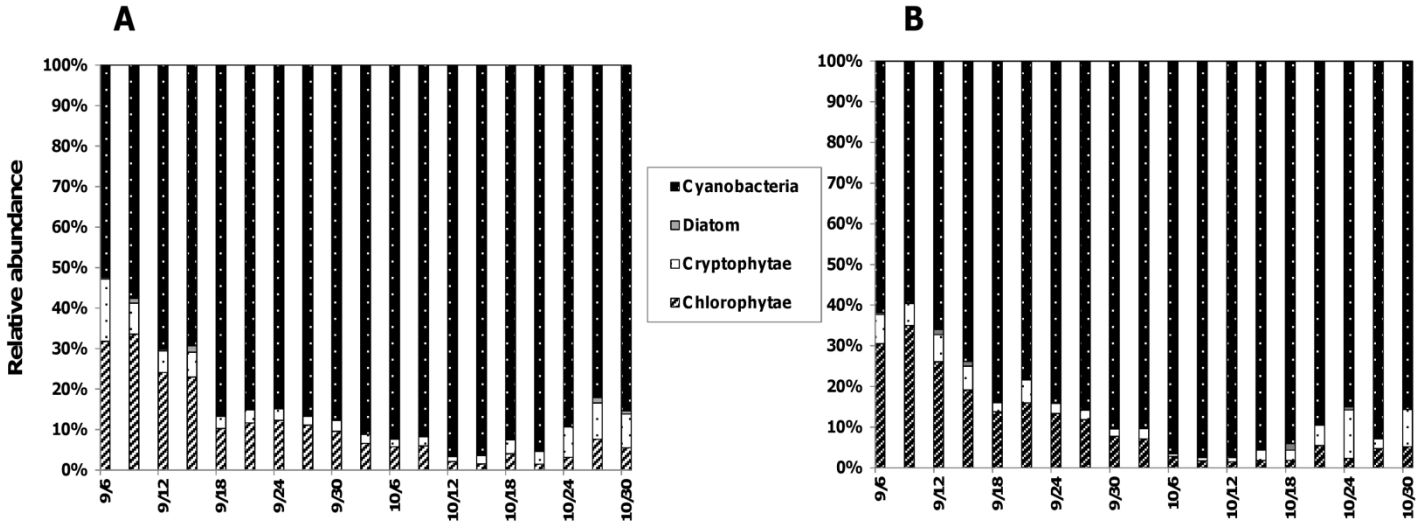

Figure 1 : Composition of phytoplanktonic community**.** Relative contributions of the different taxonomic groups within the phytoplanktonic community at 0.5 m (A) and in the depth of maximum chlorophyll, MF (B) in Lake Aydat, September 6th to October 30th 2010.

filaments, and (ii) the number of infected and non-infected cells for each filament. In addition, a minimum of 100 akinetes were inspected for the number of infected and non-infected akinetes. The original triplicates of each sample collected were analyzed. Infection parameters were calculated according to the formula proposed by Bush *et al.* (1997). These parameters include the prevalence of infection (Pr), i.e., the proportion of individuals in a given population with one or more fixed sporangia or rhizoids, expressed as Pr (%) = [(*N*i/*N*) × 100], where *N*i is the number of infected host cells (or filaments), and *N* is the total number of host cells (or filaments). We distinguished four types of infection prevalences: (i) PrC or the percentage of infected *A. macrospora* cells calculated from the total number of cells in the whole population; (ii) PrF or the percentage of infected filaments calculated from the total number of filaments, (iii) PrCF or the percentage of infected cells within those filaments which were infected, and (iv) PrAK or the prevalence of infection for akinetes. Moreover, the entire life cycle of chytrid species was described from microscopic observations and 6 tentative life stages were delineated based on phenotypic characteristics given by Canter (1954), from the younger to the more mature stage. The stage in the life cycle for each sporangium observed in our samples was assigned according to our model.

Statistical analysis

One way analysis of variance (ANOVA) was applied to test the differences between the sampling depths for the dynamics of *A. macrospora* cells and the related prevalence of infection. When significant differences were noted, a post hoc comparison (Tukey's Honestly Significant Difference [HSD], $\alpha = 0.05$) was used. Spearman's linear correlation was used to test empirical relationships between the variables under study. All statistical analyses were performed using Past software available at http://folk.uio.no/ohammer/past/ (Hammer & Harper)

Results

Physico-chemical environment

The sampling period corresponded to the seasonal cooling phase of the lake, with a weak stratification for the first sampling date (19 and 17.6°C at 0.5m and maximum fluorescence (MF) depth, respectively) that disappeared from the second date towards the end of the sampling period (10°C in both depths), although an anomaly was observed in early October. The MF depth naturally corresponded to the depth of maximum dissolved oxygen. The

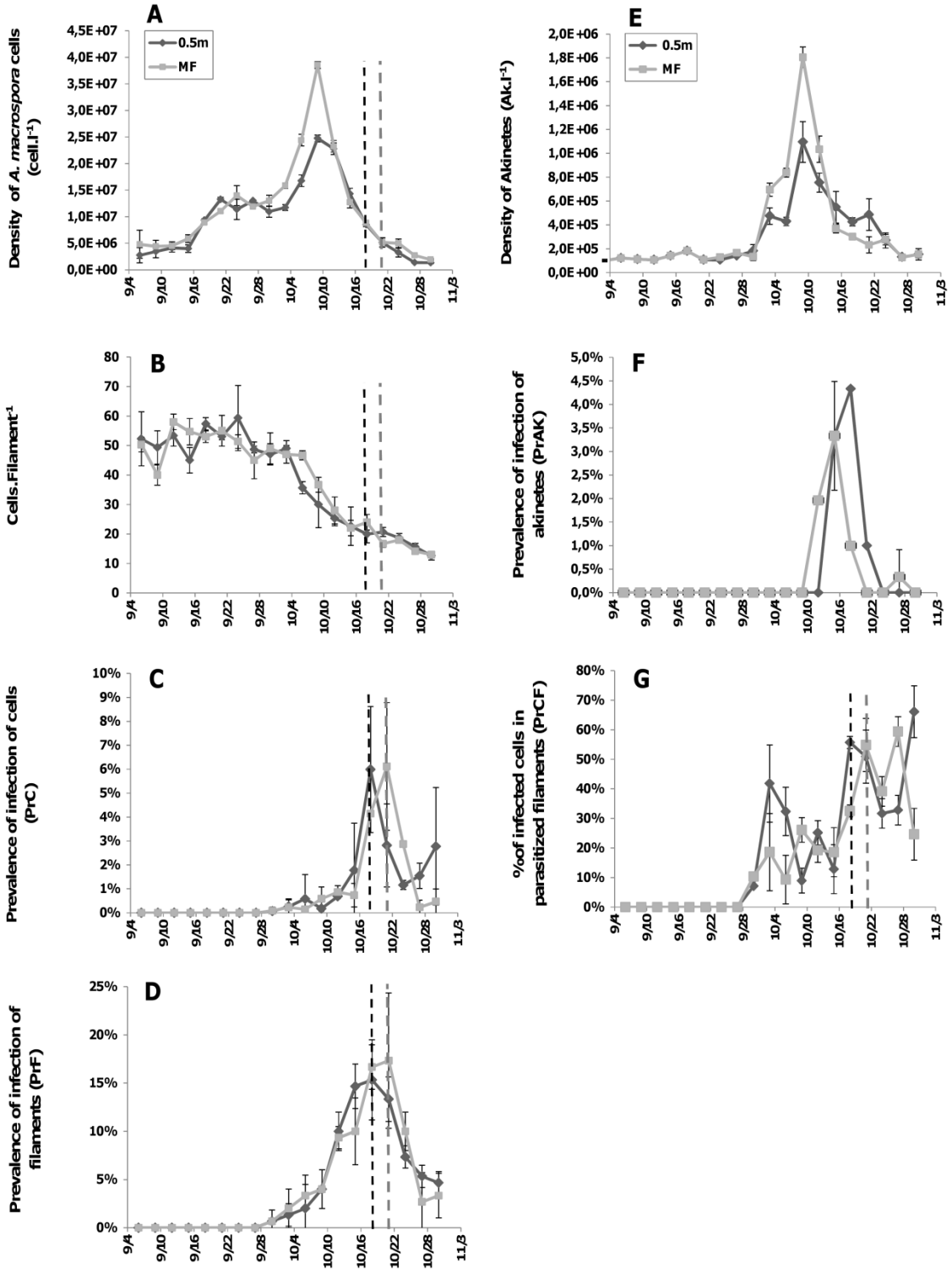

Figure 2 : Dynamics of host and chytrid parasitism parameters. Changes in the density of *Anabaena macrospora* total cells (A), cells per filament (B) and akinetes (E), and in the prevalences of infection of *A. macrospora* cells (C, PrC), filaments (D, PrF), akinetes (F, PrAK), and of cells in infected filaments (G, PrCF) at 0.5m and in the depth of maximum chlorophyll (MF) in Lake Aydat, September 6th to October 30th 2010. Vertical lines mark the transition point between the increasing and the decreasing phases in the prevalence of infection of filaments (PrF), at 0.5 (dark dashed line) and MF (grey dashed line) depths.

concentration of nutrients (nitrogen and phosphorous) showed great temporal variations (Fig. S1), but no correlation was noted between nutrient concentrations and cyanobacteria abundance.

Phytoplankton abundance

Our sampling period corresponded to the seasonal bloom of the targeted cyanobacteria *A. macrospora*, which largely dominated the phytoplankton community in the lake. Their abundances at the two sampling depths (mean for triplicates ± SD) fluctuated from 2.74 ± 0.1 to 24.7 ± 0.7 × 10^6 cells.l^{-1} and from 4.4 ± 0.1 to 38.5 ± 0.6 × 10^6 cells.l^{-1} at 0.5m and MF depth, respectively. At the beginning of the sampling period (i.e. first half of September), *A. macrospora* accounted for 52 and 62% of the total phytoplankton abundance at 0.5m and MF, respectively, values which increased to reach 96% and 98% at the bloom maximum in mid-October (Fig. 1). Accompanying phytoplankton species mainly belonged to chlorophytes (1 - 34% for the two sampled depths), cryptophytes (1-15 and 0.8-7 % at 0.5m and MF, respectively), and diatoms (0.1 - 1% for the two sampled depths). The temporal changes in the abundances of *A. macrospora* were quite similar for the two depths
sampled and exhibited 4 different growth phases: a relatively stagnant phase during the first half of September (mean growth rate, μ = 0.03 d^{-1} for the two sampling depths), a moderate increasing phase during the second half of September (μ= 0.10 d^{-1}), a more rapidly increasing phase during the first half of October when an apparent difference was noted between the two depths (μ= 0.13 and 0.15 d^{-1} at 0.5m and MF, respectively), and a decreasing phase towards the end of the study (μ= - 0.14 d^{-1} for the two sampling depths). As a consequence, the numerical abundances were similar for the two sampled depths, except during the rapidly increasing phase (i.e. from October 3rd to 9th) when they were significantly ($p < 0.02$) higher in the MF depth (maximum density 3.9 ± 0.06 ×10^7 cells.l^{-1}) compared to 0.5m (2.5 ± 0.06 ×10^7 cells.l^{-1}) (Fig. 2A). We observed that the size of the filaments of *A. macrospora* shifted significantly ($p < 0.05$) below 40-60 cells.filament^{-1} from the 3rd of October (i.e. the starting point of the rapidly increasing bloom phase) to the end of the bloom (Fig. 2B).

As with the vegetative cells of *A. macrospora*, the abundance of akinetes rapidly increased and differed from one depth to another ($p < 0.05$) during the first half of October, and then decreased towards the end of the sampling period (Fig. 2E). Maximum abundances recorded on October 9th reached 1.09 ± 0.01 and 1.81 ± 0.08 × 10^6 akinetes.l^{-1} at 0.5m and MF depths, respectively (Fig. 2E).

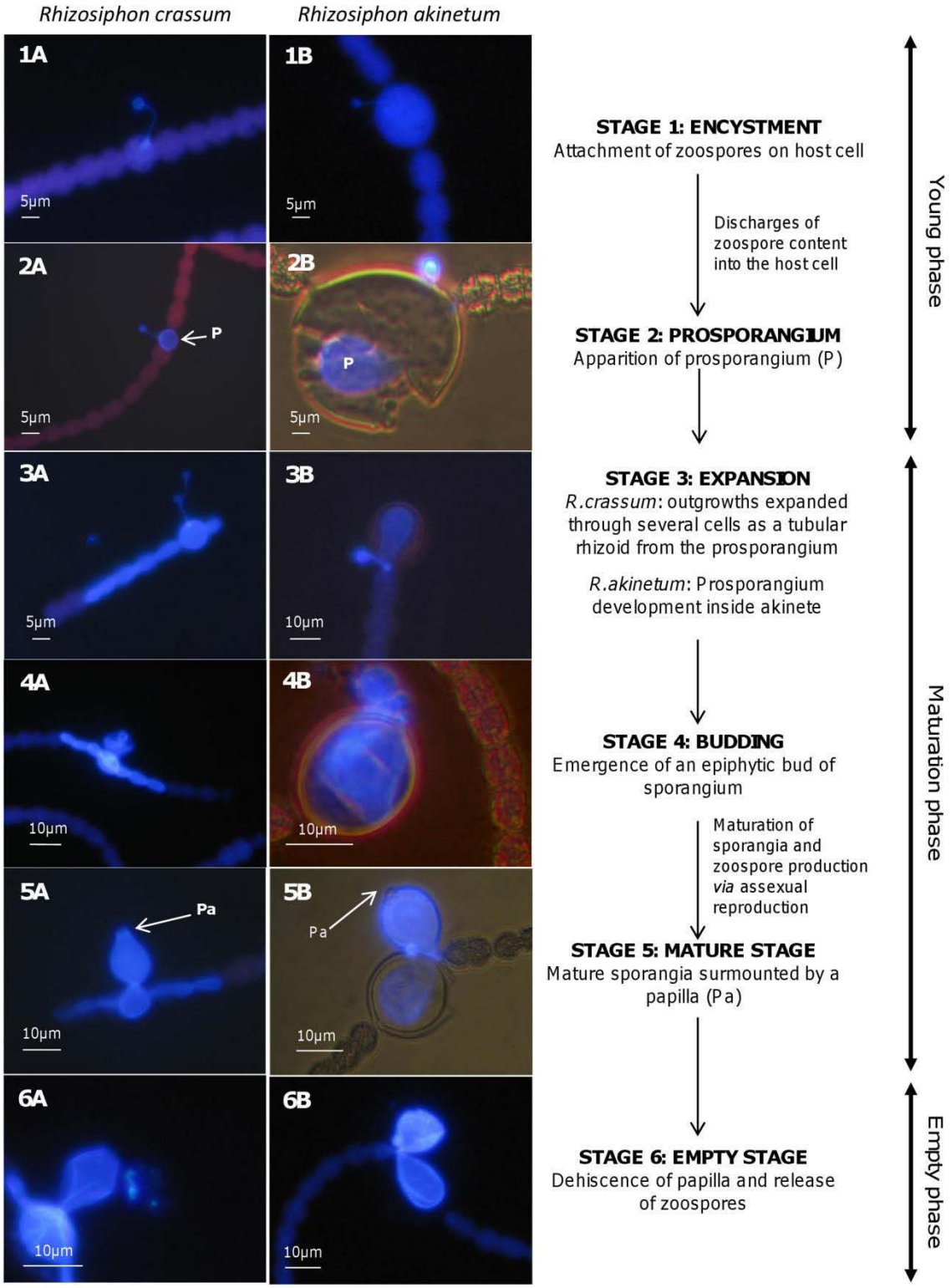

Figure 3 : Life cycles of the two chytrid species. The six different life stages of the two chytrid species, *Rhizosiphon crassum* (A) and *Rhizosiphon akinetum* (B) parasitizing the cyanobacterium *Anabaena macrospora* from the productive Lake Aydat: Stage1 : Encystment; Stage 2 : Prosporangium; Stage 3 : Expansion stage; Stage 4 : Budding; Stage 5 : Mature stage; Stage 6 : Empty stage. The six life stages were grouped into three different phases: Young phase (Stages 1 and 2), Maturation phase (Stages 3, 4 and 5) and Empty phase (Stage 6). Prosporangium (P) and Papilla (Pa).

Reconstructing the life cycle of chytrids

Based on the morphology of the sporangium and on the type of host cells, we were able to identify two species of chytrid parasites of *A. macrospora* both belonging to the same genus, *Rhizosiphon*. *R. crassum* infected both vegetative cells and akinetes by developing a tubular rhizoid system (Fig. 3$_{1\text{-}6A}$), while *R. akinetum* infected only akinetes (Fig. 3.$_{1\text{-}6B}$). We distinguished 6 tentative stages in the life cycle for these two chytrids, based on the description given by Canter (1954): Encystment, Prosporangium, Expansion, Budding, Mature, and Empty stages. Stage one, Encystment, is represented by a zoospore that has just penetrated the mucilage of a living host cell with a fine thread (Fig. 3.$_{1A}$, 3.$_{1B}$). The second stage is the prosporangium. In the prosporangium stage the contents of the zoospore are discharged into the host cell, resulting in a globose structure known as the prosporangium (Fig.3.$_{2A}$, 3.$_{2B}$). In the two chytrid species these first two stages can only infect a single host cell and thus were grouped together as "young phase" in the dynamics of life stages (see below). From the prosporangium, outgrowths, characteristic of tubular rhizoids, expands through several cells. This is the third stage: Expansion (Fig.3.$_{3A}$). The life cycle continues on to the Budding stage (the fourth stage) with the emergence of an epiphytic bud (Fig.3.$_{4A}$). This bud develops into a flask-shaped sporangium in which zoospore production occurs (asexual reproduction). It is surmounted by a gelatinous papilla typical of the mature sporangium of *R crassum* and forms the fifth stage: the Mature stage (Fig. 3.$_{5A}$). These three later stages (Expansion, Budding, and Mature stage) were grouped together in the dynamics of life stages into the "Maturation phase". The sixth and final stage of the life cycle for *R crassum* is the Empty stage (and phase) that forms after deliquescence of papilla and release of zoospores (Fig. 3.$_{6A}$). In *R akinetum*, Encystment (Fig. 3.$_{1B}$), Prosporangium (Fig.3.$_{2B}$), Expansion (Fig. 3.$_{3B}$), Budding (Fig. 3.$_{4B}$), Mature (Fig. 3.$_{5B}$) and Empty stages (Fig.3.$_{6B}$) were also observed, except that there is no tubular rhizoidal system because the infection is restricted to akinetes (Fig. 3.$_{3A}$ vs. 3.$_{3B}$).

Temporal changes in the life stages of *R. crassum*

Because the abundance of *R. akinetum* was very low (data not shown) and the first life stages of the two chytrid species were quite similar and difficult to differentiate based on their morphology, the dynamics of chytrid life stages is reported only for *R. crassum* infecting vegetative cells (Fig. 4). No differences have been reported for all stages at the two sampled depths (Fig. 4A vs 4B). Independently of the depth, abundance of sporangia increased significantly from the 3rd of October to reach its maximum values, approximately 10 fold

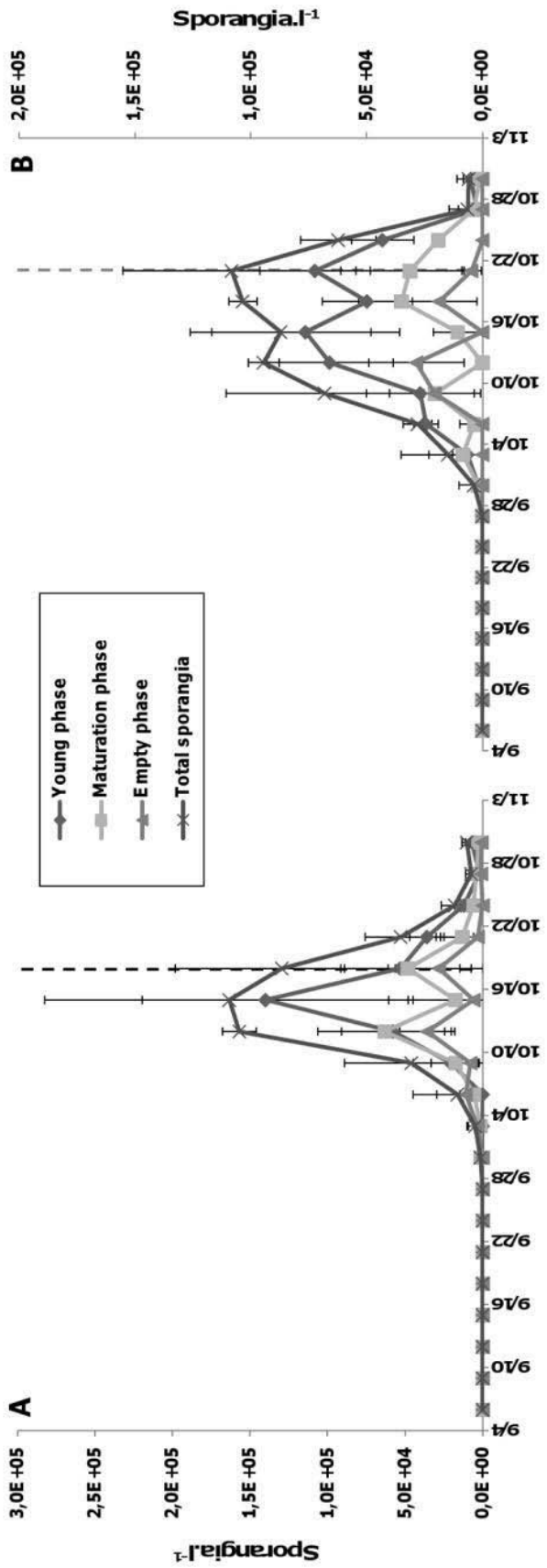

Figure 4 : Dynamics of the different phases of life cycle of *Rhizosiphon crassum*. Dynamics of the three different phases of the life cycle of the chytrid *Rhizosiphon crassum* infecting the cyanobacterium *Anabaena macrospora* at 0.5m (A) and in the depth of maximum chlorophyll (MF) (B) in Lake Aydat, September 6th to October 30th 2010. The three phases regroup the six stages of life as above: Young phase (Stages 1 and 2), Maturation phase (Stages 3, 4 and 5) and Empty phase (Stage 6) (see the main text for details). Vertical lines mark the transition point between the increasing and the decreasing phases in the prevalence of infection of filaments (PrF), at 0.5 (dark dashed line) and MF (grey dashed line) depths.

increase, on the 15th and the 21st of October (1.6± 1.1 × 10^5 and 1.08 ± 0.4 × 10^5 sporangia.l^{-1}, at 0.5m and MF, respectively). At 0.5 m, the increase in total sporangia was related to (i) the increase in young life stages which peaked on October 15th and represented 85% of total sporangia, (ii) followed by the increase in the maturation life stages plus empty sporangia between October 15th and 18th (Fig.4A). In the same way, the increasing phase of sporangia was mostly due to the young life stages which averaged 67% of total sporangia on October 21st at MF depth, enhanced by an increase in the maturation life stages plus empty sporangia between October 15th and 18th (Fig. 4B). Interestingly, for the two sampled depths, we noted that 3 days before each abundance peak of young chytrid life stages, empty sporangia and sporangia involved in the maturation process showed an increase, with their maximum abundances being systematically lower than those of young sporangia (Fig. 4).

Prevalence of infection

The infection of *A. macrospora* vegetative cells (PrC) by *R. crassum* lasted about 30 days, starting the 30th of September and involved < 1% of *A. macrospora* cells in the two depths. PrC then increased significantly ($p < 0.001$) to reach a maximum of about 6% on the 18th of October at 0.5 m and on the 21st of October at the MF depth, i.e. during the declining phase of the cyanobacterial bloom. From these dates, PrC decreased significantly ($p < 0.005$) until the end of our survey (Fig. 2C). The infection period of akinetes by *R. akinetum* lasted about 2 weeks, roughly between October 12th and 24th, with maximum prevalence of 4.3 and 3.5% at 0.5m and MF depths, respectively (Fig. 2F).

Changes in the infection prevalence in the filaments (PrF, i.e. percentage of infected filaments calculated from the total number of filaments) were similar to those in the whole *A. macrospora* population (i.e. PrC), including the maximum values which were observed on October 18th at 0.5m and on October 21st at MF (Fig.2d). Nevertheless, the values of the infection prevalence for filaments were higher than those obtained for the whole population, and reached 15 ± 3.1% at 0.5m and 17 ± 4.2% at MF. PrF then decreased rapidly and significantly ($p < 0.05$) towards the end of the sampling period (Fig. 2D).

Within a filament, the percentage (0 to 66 ± 9 %) of infected cells to the total cell number, i.e. PrCF, were in the same range and fluctuated similarly in both depths (Fig. 2G). Fluctuations in PrCF were consistent for the two depths but, overall, roughly increased during the whole infection period, i.e. from September 30th to October 30th. However, when averaged PrCF values in Figure 2G for two critical periods, i.e. the increasing and the decreasing periods

for both PrC and PrF (i.e. Fig. 2C,D), the mean PrCF values for the increasing period (21 ± 11 and 19 ± 6% at 0.5m and MF, respectively) were significantly ($p < 0.05$) lower compared to those (47 ± 10 and 45 ± 12%) calculated for the decreasing period. Clearly, the number of infected cells within those filaments, which were infected, was significantly higher during the declining phase of *A. macrospora* bloom, compared to the growth phase.

Discussion

General considerations. This study constitutes an original report on fungal parasitism associated to a complete cyanobacterial bloom event in a natural aquatic ecosystem. Our data contribute significantly to a better understanding of both the life cycle of chytrids in natural environment and their infection strategies. Although our species identification was solely based on morphological traits (Canter, 1951, Canter, 1954), the data in our study highlight that, at the natural community level, a single cyanobacterial host species (*Anabaena macrospora*) can offer different cellular niches for two parasites in the Chytridiomycota (i.e. chytrids): *Rhizosiphon crassum* and *Rhizosiphon akinetum*. Similarly, a previous study has shown that another freshwater *Anabaena*, *A. smithii*, could also be parasitized by two different chytrid species which were able to infect akinetes and heterocysts. However, the identity of the chytrids in the study by Takano *et al.* (2008) in Lake Shumarinai, Hokkaido, Japan, was not determined. In general, *Rhizosiphon* species are well known as chytrid parasites of cyanobacteria and, more particularly, of the genus *Anabaena* (Canter, 1951, Sparrow, 1960, Canter, 1972). The possibility that the two species of *Rhizosiphon* infecting the same host represent cryptic forms of the same species, e.g. based on the as yet unavailable molecular sequences, remains an interesting open question.

The duration of the life cycle of chytrid parasites from a field study point of view. We have identified six different phenotypic life stages (Canter, 1954) for *R. crassum* and *R. akinetum* due to a high sampling resolution coupled to an improved staining technique (Rasconi*, et al.*, 2009) and have provided a tentative full description for *R. crassum*. We have grouped these six stages in the life cycle into three phases (young, maturation and empty phase) corresponding to the growth phases (i.e. encystement, germination, growth and maturation) known from the general life cycle of the Chytridiomycota. In our field survey, the young phases were followed within a period of three days by empty phases, and so we were able to infer that the complete life cycle of *R. crassum* lasted about 3 days in natural conditions.

Cultivation experiments could give more precise information on optimal conditions of growth. Nevertheless, our field results seem to be in agreement with results obtained for other parasitic chytrid species maintained in laboratory conditions. Indeed, Bruning and Ringelberg (1987) calculated that *Rhizophydium planktonicum*, a typical chytrid parasite of diatoms, accomplished a full life cycle for about 2 days in optimal conditions. Recently, Berger *et al.* (2005) described the life cycle of *Batrachochytrium dendrobatidis*, one of the most deadly contemporary skin disease agent that drives the decline of amphibian populations worldwide, and suggested that the time for completion of the life cycle was between 3 and 5 days. From these comparisons, it is thus likely that our sampling resolution was high enough to determine the generation time of the parasitic chytrids under study during the seasonal bloom of *A. macrospora*.

One genus, two species, and different strategies for infection. The main differences in strategies used by the two chytrid species were the type of targets (host cells) in *A. macrospora* filaments, and the methods of parasitic exploitation of these cells. Infectivity of *R. crassum* was observed throughout the whole filament, with rhizoids crossing through both vegetative cells and akinetes, whereas that of *R. akinetum* was highly specific and restricted to akinetes. These differences suggest the co-occurrence of different infectivity strategies, depending on the type of cellular niches offer by the hosts and, we suspect, on the availability of energy required for the parasite development. Because of its capacity to infect several cells at the same time, *R. crassum* could be considered as having access to more energy than *R. akinetum*. However, there might be an energetic advantage to infect akinetes. Compared to vegetative cells, akinetes are known to contain approximately 2-fold more carbon (Sutherland*, et al.*, 1979), and 16-fold more glycogen which constitutes the prime energy reserves of zoospores for dispersal (Suberkropp & Cantino, 1973, Gleason*, et al.*, 2008). This indicates that the zoospores of *R. akinetum* could have access to the same or even more energy than those of *R. crassum*, in spite of the restricted to akinetes. However, akinetes were not present during the entire bloom event and presented a systematically lower abundance than vegetative cells (i.e. 10 to 150 fold). Because chytrid infection is generally a host density-dependent process (Ibelings*, et al.*, 2004), we consider that the ability of *R. crassum* to infect both vegetative cells and akinetes could be an advantage in our case study. The type of cells within the same host species could thus influence the process of chytrid infection in natural communities of filamentous cyanobacteria, depending on the resource availability for spreading infection and prevalence.

The time necessary to complete the life cycle of zoosporic true fungi is short and their dissemination phase is known to be highly dependent on the host density (Fuller, 1986, Bruning, 1991, Kudoh & Takahashi, 1992, Piotrowski*, et al.*, 2004). In our case study, infections seemed to start from a minimal host density threshold of 1.5×10^7 vegetative cells liter^{-1} and 1.8×10^6 akinetes liter^{-1} for *R. crassum* and *R. akinetum*, respectively (Fig. 2). This indicates that chytrid infection could be promoted at relatively low host densities in natural conditions (Kagami & Urabe, 2002, Alster & Zohary, 2007). Nevertheless, detailed analysis of chytrid infection revealed that the maximum prevalence was mainly due to the increase of infected cells within the already infected filaments (Fig. 2D). These results imply that, at the end of the bloom event, some trichomes could be intensely infected while others were completely healthy, which was observed during the end of our survey, suggesting a coexistence of resistant and susceptible *A.macrospora* filaments to the fungus attacks. It is well known that inside a host population, some genotypes are more susceptible to parasitism than others (Thompson & Burdon, 1992, Henter & Via, 1995, Carius*, et al.*, 2001). Recently, Sonstebo & Rohrlack (2011) demonstrated a close relationship between genotypes, chemotypes and the severity of chytrid infection for strains of the cyanobacteria *Planktothrix*. Furthermore, other authors have indicated chemotactism as one mechanism to explain the attraction between hosts and chytrids in environmental samples (Powell, 1994, Moss*, et al.*, 2008, Gleason & Lilje, 2009). In the case of decreasing host cell density such as during the late bloom phase when the maximum prevalence was recorded in our study, we suggest that newly produced zoospores could be more attracted by the chemical cues from the nearby non-infected cells located within infected filaments, compared to those cells in non-infected filaments located too far away for an efficient chemotactic detection. This may help explain the paradox of the co-occurrence of low cellular density of *A. macrospora* and low infection prevalence of filaments, with a high number of infected cells within parasitized filaments at the end of the bloom (Fig.2A,E,F). This could also be explained by a simple opportunistic development (i.e. of both parasites and saprotrophs) on moribond host individuals in the declining bloom phase. However chytrid infection started when cyanobacteria population was in a rapidly growing phase characterized by (i) an enhanced increase in cellular density, (ii) high chlorophyll content as attested by high autofluorescence of cells (Fig. 3.$_{2A}$), and (iii) high proportion of cells located above the limit of euphotic layer for the two sampling dephts. These conclusions tend to confirm previous observations that chytrids parasitize healthy hosts

and thus impact directly the dynamics of cyanobacterial populations (Van Donk & Ringelberg, 1983, Sen, 1987, Holfeld, 1998, Ibelings*, et al.*, 2004).

Parasitism by chytrids has direct and indirect effects on filamentous cyanobacterial blooms. Parasitic chytrids derive their growth energy from the host cells, a situation that may trigger the death of the latters. Parasites can also reduce the fitness of their host, or allow infected hosts to remain strong competitors (Vogwill*, et al.*, 2008). These direct effects of parasites could vary greatly with space and time. In this study, the infection prevalence peaked at 20% of total filaments, which is considerably lower than the value of 98% reported in 2007 in the same lake for the same host species (Rasconi*, et al.*, 2012). In Shearwater Lake, Wiltshire, United Kingdom, Sen (1988) also reported a significant difference in the severity of chytrid infection between successive years, during the summer-autumn development of the cyanobacteria *Microcystis aeruginosa.* Nevertheless, in these last two studies which centered on the host genera *Anabaena* and *Microcystis*, abiotic factors and host density were quite similar and failed to empirically explained the interannual differences in the chytrid infectivity during seasonal cyanobacterial blooms. It was reported that cyanobacterial blooms
often are the result of the growth of a few dominant genotypes (Sabart*, et al.*, 2009), which may change from year to year in a same lake (Tanabe & Watanabe, 2011). Sonstebo and Rohrlack, (2011) have emphasized that chytrid virulence was strain dependent. Thus, interannual shift in genotypic composition of host population could lead to different sensitivities to parasitism. The sensitivity to fungal parasitism for the dominant genotype can be quite high and contribute significantly to accelerate the decline of the blooms with liberation of niches for other species (Van Donk, 1989) or, conversely, can be low and weaken the top-down effect of chytrids on a restricted fraction of cyanobacterial population.

Besides their role in maintaining phytoplankton diversity, chytrids also infect resistance forms (i.e akinetes) during the pelagic phase of cyanobacteria. Akinetes are key elements that promote the seasonal germination of cyanobacterial filaments when favorable conditions for growth return (Baker & Bellifemine, 2000). In this study, we were able to observe that up to about 5% of akinetes were infected by their specific *R. akinetum* chytrid parasites. Some cyanobacterial filaments could thus be devoid of their storage cells (Wood*, et al.*, 2009). In this way, we can suggest that the specific parasitism on akinetes could lower or delay the seasonal growth of cyanobacterial in succeeding year, although this hypothesis remains to be experimentally tested.

In addition to the above direct top-down impact on cyanobacteria communities, chytrid infection also affects the integrity of filaments and could reduce their size (Sigee*, et al.*, 2007). In a case of filamentous species, the cell-cell adhesion described by Flores and Herrero (2010) is fundamental to the transfer of compounds essential for a healthy filament from cell to cell. By their parasitic action, chytrids kill their host cells. The death of cells inside a filament could induce the fragility of filaments, and impact the adhesion mechanism, which could result in the breaking of cyanobacterial filaments. This may help explain why, during our survey, the significant ($p < 0.001$) reduction of the filament size which decreased from 52 to 10 cells filament^{-1} was significantly correlated with the increasing infection prevalence in the two sampling depths ($rs = -0.79$ and -0.84, $p<0.001$, for 0.5 m and at MF depth, respectively). Because the availability of filamentous cyanobacteria to grazing is mainly constrained by their inedible size (Lampert, 1987, Gliwicz, 1990), we hypothesize that the "mechanistic fragmentation" of cyanobacterial filaments in small units by chytrid parasitism may increase the availability of cyanobacteria to grazers, and so, accelerate the decline of cyanobacterial blooms.

Concluding remarks. In this study, we were able to describe two apparent chytrid species with similar life cycles but with different strategies for infestion in the blooming filamentous cyanobacterial host *A. macrospora*, monitored using high sampling resolution in a productive freshwater lake. One chytrid species infected both vegetative cells and akinetes and was responsible for the death of cells within host filaments, while the other species infected only akinetes and may thus affect the survival of cyanobacteria hosts and their proliferation from year to year. We propose that with maximum prevalence levels, which increased from 4-6% of total cells and akinetes, 17% of total filaments, and >60% of total cells in infected filaments, chytrid parasitism is one of the driving factors involved in the decline of cyanobacterial blooms. In addition, by the so-called "mechanistic fragmentation" of cyanobacterial filaments, chytrids could weaken the resistance of *A. macrospora* to grazing, which could further accelerate the decline of blooms. This adds to the roles of chytrid zoospores which are well known to upgrade the biochemical diet of zooplankton (Kagami*, et al.*, 2007, Masclaux*, et al.*, 2011), establishing zoosporic fungi as potential key players in the food web dynamics. Our conclusions are empirical and mainly based on the results from field observations that require experimental validation. Furthermore, our study was conducted during a single bloom event and in one temperate lake. Clearly, although *A. macrospora* blooms are an annual event in Lake Aydat which we investigated, repeating a similar study

for different bloom species, over several years, and on a wide geographical scale, remains necessary for accurate generalization.

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Article 4 :

Spatiotemporal distribution of aquatic fungal parasitism: the case of chytrid-cyanobacterium pairings

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Article en preparation

Introduction

The phytoplankton is the most abundant primary producer in freshwater systems as such it constitutes a key player in the trophic food web. The structure, diversity, and seasonal succession of phytoplankton community have been largely discussed previously (Sommer*, et al.*, 1986, Beisner*, et al.*, 2006, Anglès*, et al.*, 2008, Rychtecký & Znachor, 2011). Phytoplankton seasonal succession is driven by several biotic factors such as the predation (Sarnelle, 2005, Masclaux*, et al.*, 2012), the competition among themselves (Sommer, 1989, Yoshiyama*, et al.*, 2009) or with other food web components (Bratbak & Thingstad, 1985), and the parasitism (Caiola & Pellegrini, 1984, Pollard & Young, 2010, Rasconi*, et al.*, 2012), although the later factor remains largely understudied. The most common pathogens of the freshwater phytoplankton are probably fungal species that belong to the phylum Chytridiomycota (i.e. chytrids) (Gleason*, et al.*, 2008). These fungi are involved in the structuring and temporal succession of the phytoplankton community by promoting the decline of host phytoplankton species (Van Donk, 1989, Kagami*, et al.*, 2007). Recently, few studies have investigated the chytrid diversity (Lepère*, et al.*, 2008) and its vertical (Lefèvre*, et al.*, 2008), horizontal (Monchy*, et al.*, 2011) or temporal (Rasconi*, et al.*, 2012) distribution in freshwater lakes, at the community level. However, although parasitic chytrids are totally dependent to specific hosts, to the best of our knowledge, only one study dealt with the spatiotemporal distribution of targeted chytrid-phytoplankton pairing (Gsell*, et al.*, 2012). In this study authors have investigated the vertical distribution of the couple *Asterionnella Formosa - Zygorhizidium planktonicum,* during 1.5 years, in the Lake Maarsseveen,

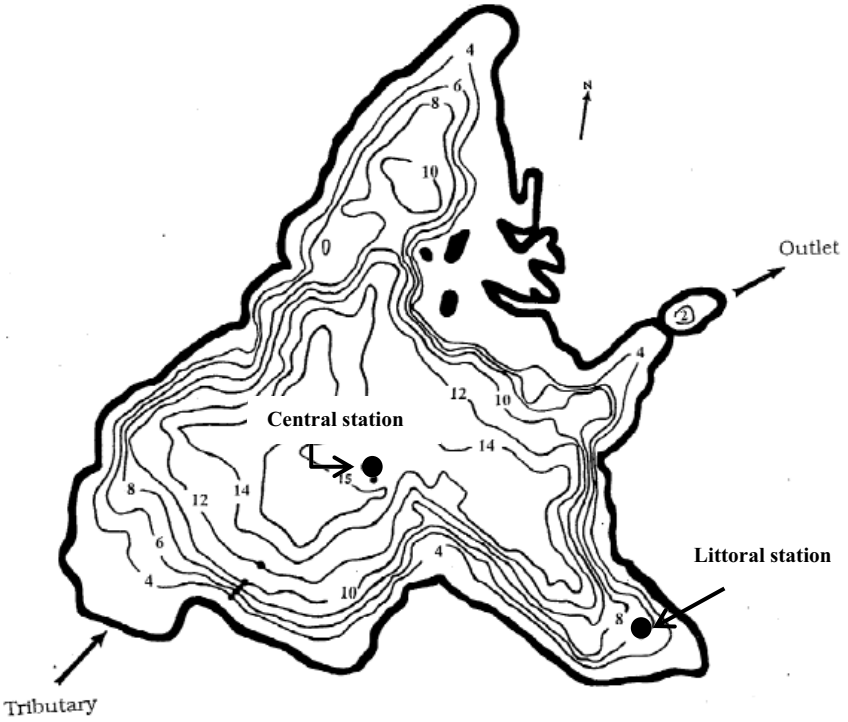


Figure 1 : The bathymetric map of Lake Aydat (from Rabette and Lair, 1999) display the location of the two sampling stations: Central and littoral stations. On the bathymetric map, the arrows show the flux of water and the numbers indicate the depth in meter.

Nederland. Gsell, *et al.* (2012), revealed that both vertical and temporal gradients of abiotic factors could impact host-parasite interactions. However, in most freshwater lake studies on the host-parasite interactions (Kagami & Urabe, 2002, Ibelings*, et al.*, 2011, Rasconi*, et al.*, 2012), the central area of lake was the only monitored point. Consequently, the horizontal host-parasite pairing distribution has never been taking under consideration. Nonetheless, phytoplankton blooms, especially surface bloom such as cyanobacterial blooms are known to be wind-driven, and so with a patchy horizontal distribution.

In order to provide an accurate host-parasite pairing distribution and to gather information on the design of sampling strategy used to survey cyanobacteria-chytrid pairings in eutrophic lake, we investigated the spatiotemporal variations of cyanobacteria-chytrids interactions by studying vertical, horizontal and temporal distribution of (i) host population, (ii) prevalence and intensity of chytrid infection and (iii) chytrid fecundity, during the bloom of the cyanobacterium *Anabaena macrospora* in the eutrophic Lake Aydat, France.

Materials & Methods

Study site and sample collection.

Samples were collected in Lake Aydat (45°39'48''N, 002°59'04''E), a small eutrophic lake (Zmax = 15 m, surface area = 60 ha) with a large catchment area (3 x 10^4 ha), which is located in the French Massif Central region. To investigate a spatiotemporal variation in host-parasite pairing involving the Chytridiomycota *Rhizosiphon sp.* and the cyanobacterium *Anabaena macrospora* (syn. *Dolychospermum macrosporum*), Lake Aydat was sampled.

Based on earlier works (Paterson, 1960, Ibelings*, et al.*, 2004, Gerphagnon*, et al.*, 2013) we sampled at three strategic dates: (i) during the increasing phase of the cyanobacterial bloom known as the phase when chytrid infection starts (7th of October 2011), (ii) when the cyanobacterial bloom peaked (14th of October 2011), coinciding with the increasing fungal infection, and (iii) during the decline of the cyanobacterial bloom (21st of October 2011) when the chytrid infection peaks . For each date, two stations were sampled at the central and the littoral areas of the lake (Fig 1). The central point corresponds to the area of maximum depth, whereas the depth of the littoral sampling station was 5m. For each date, the center of the lake was sampled at 5 different depths (0.5, 2, 4, 6 and 8m), and the littoral at 3 different depths (0.5, 2 and 4m). For each sampling depth, twenty liters of lake water were

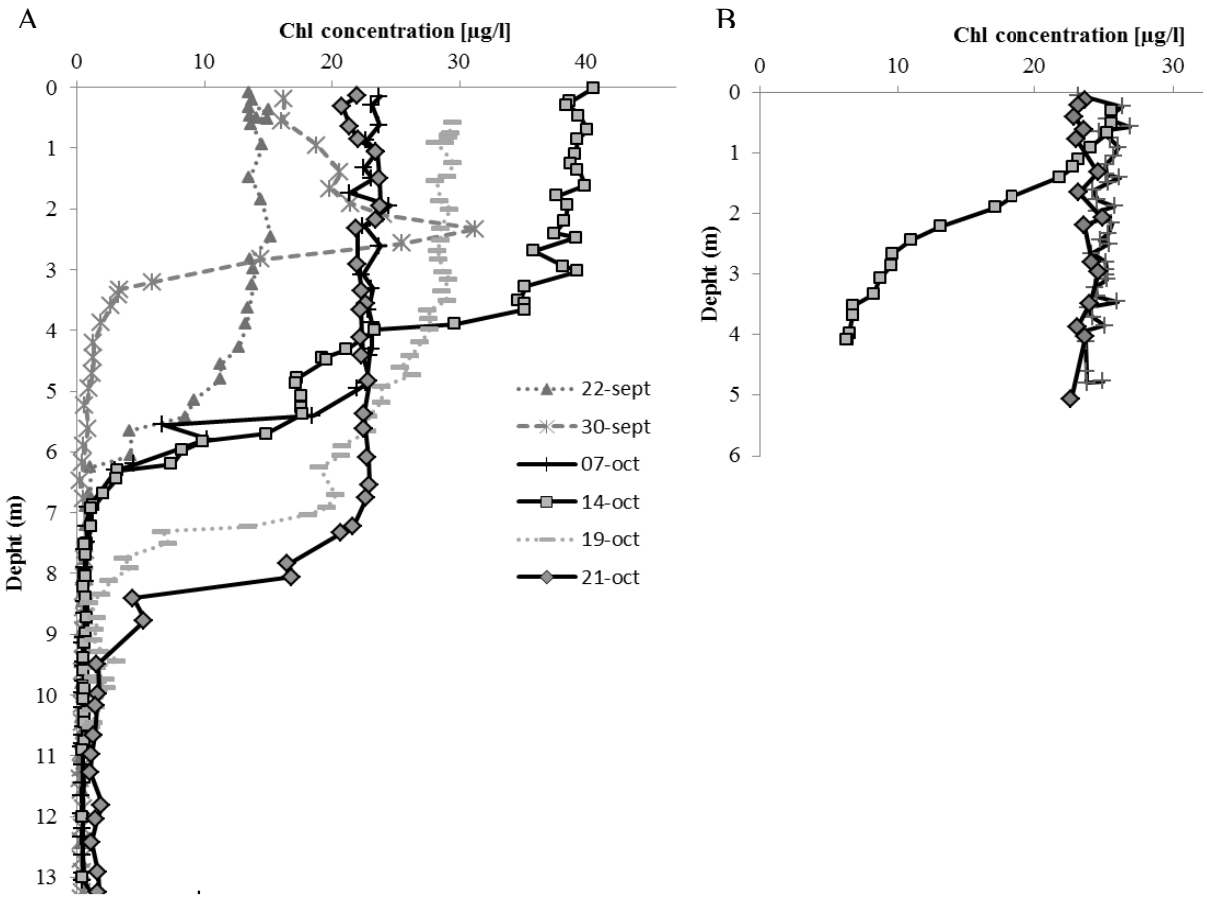


Figure 2: Chlorophyll concentration profiles (from cyanobacteria) in October 2011 from the center (A) and the littoral (B) stations of lake Aydat. Black lines represent sampled dates (7th, 14th and 21st of October 2011), others are intermediate dates (22nd and 30th of September and 19th of October 2011).

sampled using an 8-L Van Dorn bottle. To eliminate the metazoan zooplankton, immediately after being collected the samples were prefiltered through a 150 µm-pore-size nylon filter, poured into clean transparent recipients, and then transferred to the laboratory for processing. The >150-µm fraction was checked to make sure that it did not contain any cyanobacterium. Back in the laboratory, samples were treated: *i)* to study the host community (triplicate180-ml aliquots of the raw samples were fixed with Lugol's iodine), *ii)* to investigate the prevalence the intensity of infection and the chytrid fecundity using our double staining method (Gerphagnon*, et al.*, 2013).

Physico-chemical parameters

For each sampled depth and station, water transparency was measured *in situ* using a Secchi-disk (Z_s) and the depth of the euphotic zone (Z_{eu}) was calculated according to Reynolds (1984): $Z_{eu}=1.7\times Z_s$. Temperature and dissolved oxygen profiles were obtained using a multiparametric probe ProOdO^{TM} (Ysi, Germany). A vertical pigment profile was obtained by using a BBE Fluoroprobe® (Moldaenke, Germany) (Fig. 2).

Host community analysis

Triplicate 180-ml aliquots of raw samples were fixed with Lugol's iodine. For each replicate, 5 to 20 ml (depending on the phytoplankton density) were allowed to settle overnight in a counting chamber. The cells were then counted under an epifluorescence microscope (Zeiss Axiovert 200M) following the classical Utermöhl method. The entire counting chamber was inspected and *A. macrospora* filaments, vegetative cells and akinetes (immature and mature) were quantitatively analyzed. The distinction between mature and immature akinetes was based on their morphology (the presence of an outer envelope layer is characteristic of mature akinetes), shape (mature akinetes are ovoid whereas immature akinetes are spherical) (Sutherland*, et al.*, 1979), and size (16-23µm width and 21-28µm length for the ovoid mature akinetes *vs* 13-17µm diameter for spherical immature akinetes).

Chytrid parasitism

For chytrid infection parameters, samples were treated following the size-fractionated community method developed by Rasconi *et al*. (2009). Briefly, 18L of sampled water was concentrated on 25µm pore size nylon filter. Large phytoplankton cells (≥ 25µm), including the filamentous cyanobacteria *A. macrospora*, were collected by washing the filter with

0.2µm-pore-size-filtered lake water, fixed with formaldehyde (2% final concentration), and an aliquot of 195µl was stained for the chitin wall and the zoospore nuclear genomic DNA of fungi. To explore these two parameters simultaneously, we used our double staining method (Gerphagnon *et al.* 2013) which consists of an incubation of our aliquot during 55 min with SYTOX-green (0.1µM final concentration), before adding CFW (final concentration 2.5%, vol/vol) followed by an additional 5 min incubation. To prevent bleaching, 5µl of an anti-fading solution (4:1 mixture of Citifluor and VectaShield; Vector Laboratories) was added before mounting the samples between glass slides and cover slips. The chitin walls stained with CFW were examined using UV excitation (405 nm); zoospore nuclear genomic DNA stained with SYTOX-green was explored under blue light illumination (488nm). We carried out the observations under an inverted epifluorescence microscope Zeiss Axiovert 200M at ×400 magnification. For each mature stage encountered, the fecundity of the chytrid was investigated by analyzing the zoosporic content from the various different images acquired at intervals of 0.8 to 1.1µm and then analyzed using Axiovision 4.1 software.

We systematically inspected 200 filaments, comprising 2480 to 4996 individual cells of *Anabaena macrospora* to determine the number of infected and non-infected vegetative cells and filaments. In addition, we inspected 300 mature akinetes for the number of infected and non-infected akinetes. Each sample was analyzed in the original triplicates collected. Infection parameters were calculated according to the formula proposed by Bush *et al.* (1997). These parameters include the prevalence of infection (Pr), i.e., the proportion of individuals in a given population with one or more fixed sporangia or rhizoids, expressed as Pr (%) = [(*N*i/*N*) × 100], where *N*i is the number of infected host cells (or filaments or akinetes), and *N* is the total number of host cells (or filaments or akinetes). The second parameter is the mean intensity of infection (I) calculated as I= Np/Ni, where Np is the number of parasites, and Ni the number of the infected individuals within a host population.

Moreover, for each chytrid encountered, its life stage (stage 1 to 6) was noted as described in Gerphagnon *et al.,* (2013). For each mature and empty sporangium, the biovolume of the sporangia was calculated by assimilating sporangia to spheres (Hillebrand*, et al.*, 1999). From the biovolume of mature and empty sporangia, we calculated the theoretical zoosporic content by using the Conversion Factor (CF) of 0.0172 zoospores per μm^3 of sporangium of *Rhizosiphon akinetum* determined for this species in a previous study (Gerphagnon*, et al.*, 2013). This theoretical zoosporic content was compared to the observed zoosporic content from the various different images acquired as described above.

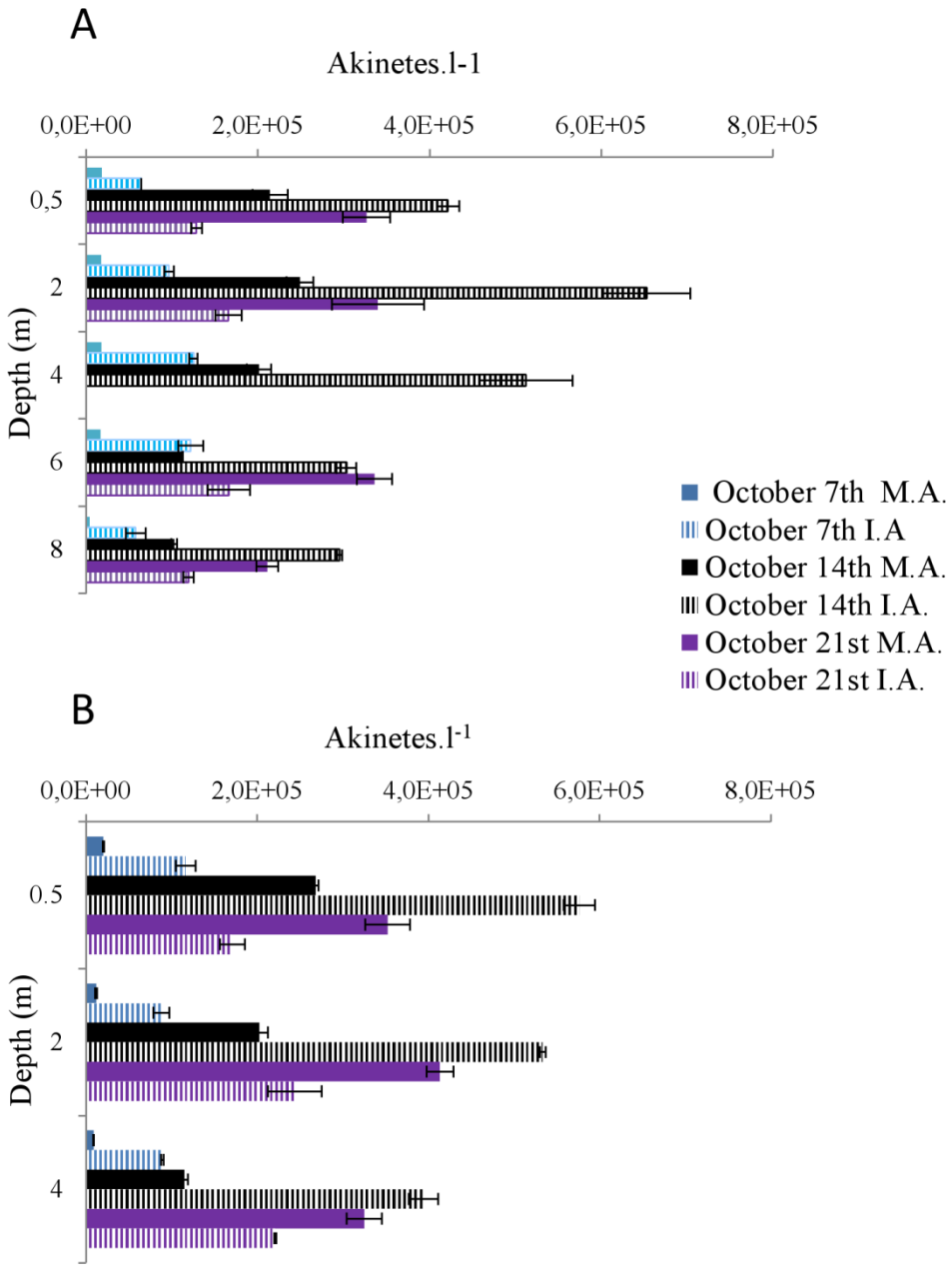


Figure 3: Variations of akinete abundances observed at the central (A) and littoral site (B) the 7th (blue), 14th (black) and the 21st (violet) of October 2011. Mature akinetes (M.A) were represented by solid bars and immature akinetes (I.A) were represented by hatched bars. The error bars indicate the standard deviation.

Statistical analyses

The non-parametric Kruskal-Wallis test was used to test effect the spatial (vertical and horizontal) variations of each variable. Pairwise comparisons of these distributions were computed with Kolmogorov–Smirnov tests. All statistical analyses were conducted using PAST.

Results

Physico-chemical environment.

Whatever the date, both sampling sites presented the same water temperature for a specific depth. Thermocline in the central station was located at 6m the first two dates (7th and 14th of October) and at 8m the last one (21st of October). The temperature of epilimnion decreased during the sampling period from 15.8±0.2°C the 7th of October to 12.4±0.2°C the 21st of October, with a larger decline between the 14th and the 21st of October (approximately 3°C). Similar to temperature, oxygen concentrations were closed between sites for a given depth and showed a clear stratification. They were homogeneous on the first 5 meters (the 7th and the 14th October) with an average value of 9.97±0.2 mg.l^{-1}, and on the first 6 meters for the last sampled date showing a lower value of 8.8±0.2 mg.l^{-1}. The last part of the water column show a rapid decrease to values close to 0mg.l^{-1} whatever the sampling date was. The euphotic layer fluctuated from 2.16 m (October 14th) to 2.7m (October 7th and 21st) in the center of the lake and from 0.8m (October 14th) to 2.7m (October 7th and 21st) in the littoral site.

Host community

Our sampling period corresponded to the seasonal bloom of the targeted cyanobacteria *A. macrospora* which largely dominated the phytoplankton community, accounting from 78.6±0.03% up to 87.4–±0.02% (i.e. mean for triplicates ± SD) of the total abundances of phytoplankton recorded on October 7th and October 14th, respectively. Overall, the inter-sites comparison of vegetative cell densities did not show any significant difference excepted on the 14th of October when the central station presented significantly higher abundances ($p=0.007$). The maximum abundances of vegetative cells of *Anabaena macrospora* were reported on the 14th of October, for both sites (2.38±1.01×10^{7} and 1.56±0.2×10^{7}cells.l^{-1}, for central and littoral sites, respectively). For the two first sampling dates, the abundance of vegetative cells decreased significantly with the depth ($p<0.05$) and the maximum abundances

were recorded at 2m in the central site ($2.33\pm0.2\times10^7$ and $4.18\pm0.5\times 10^7$ cells.l^{-1} for the 7th and the 14th of October, respectively) and at 0.5m for the littoral site ($1.9\pm0.1\times10^7$ and $1.88\pm0.2\times10^7$ cells.l^{-1} for the 7th and the 14th of October, respectively). On the last sampling date (21st of October), cyanobacterial bloom declined and vegetative cells presented significantly lower abundances for both sites ($p=0.018$). As a consequence of the bloom decline, the maximum abundances of the vegetative cells on 21st of October were recorded deeper (i.e. compared to the other sampling dates) in the water column, at 6m in the central station ($1.7\pm0.3\times10^7$ cells.l^{-1}) and at 4m in the littoral station ($1.23\pm0.07\times10^7$ cells.l^{-1}).

Because the akinetes of *A. macrospora* presented two distinct stages of development, we decided to separate immature akinetes (I.A.) and mature akinetes (M.A.). Globally, no significant difference was noted between sites for the distribution of these two "populations". Concerning the two first dates, whatever the stations and depths, I.A. presented significantly higher abundances (Kruskal-Wallis test, $p<0.05$) (Fig.3). During the sampling period, M.A. abundances increased significantly (Kruskal-Wallis test, $p=0.018$) and presented significant higher abundances than I.A. the 21st of October (Kruskal-Wallis test, $p<0.02$). At this date M.A. presented average abundances of $3.64 \pm 0.4\times10^5$ and $3.03\pm0.6\times10^5$ akinetes.l^{-1} for the littoral and central site, respectively (Fig.3). Concerning their vertical distribution, akinetes densities decreased all along the water columns for each date and station, excepted for the 7th and 21st of October at the central site when their densities were higher and homogeneous on the first 6 meters and decreased only on the deepest depth (Fig 3).

Chytrid parasitism

Based on the morphology of the sporangium and on the type of host cell, we were able to identify two species of chytrid parasites of *A. macrospora: Rhizosiphon crassum* and *Rhizosiphon akinetum. R. crassum* was reported on vegetative cells and develops a tubular rhizoid system infecting several vegetative cells, whereas *R. akinetum* infected mature akinetes and was restricted to them (Gerphagnon*, et al.*, 2013). In addition to their different type of host cells, these two species presented significant differences in term of both prevalence of infection and abundance, with a maximal abundance of *R. crassum* approximately 11 fold lower than that recorded for *R. akinetum.*

R. crassum infected a maximum of $0.68\pm0.04\%$ of vegetative cells with a maximum abundance of $0.18\pm0.08\times10^5$ sporangia.l^{-1} recorded the 7th of October at 2m in the central site. Because all life stages of *R. crassum* were not represented and the very low levels of its

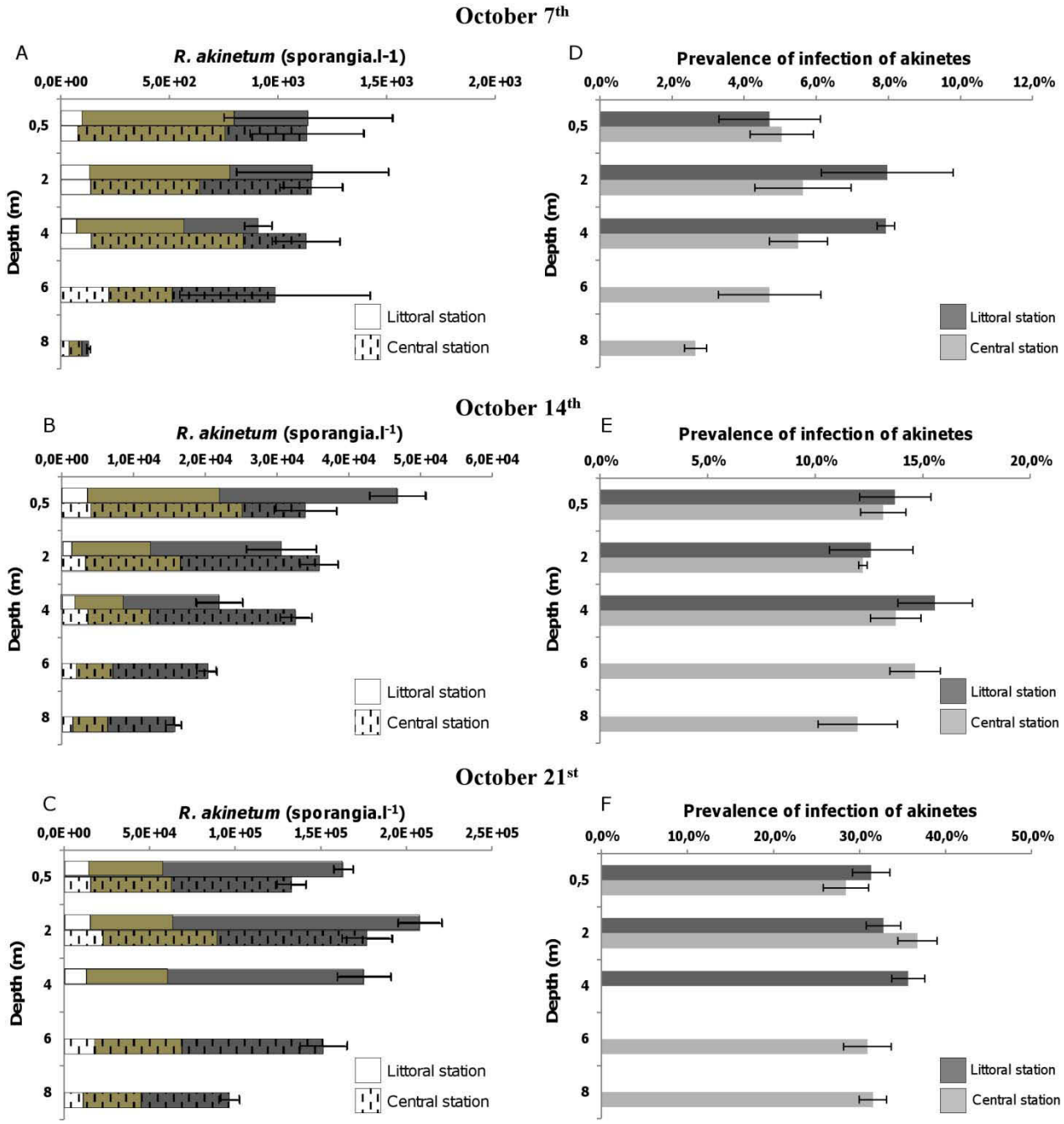


Figure 4: Vertical variations of *R. akinetum* abundance for each phase of its lifecycle (Young phase (grey), Mature phase (brown), Empty phase (white)) (A,B,C) and of prevalence of infection (D,E,F) due to this fungus species the 7th (A, D), 14th (B, E) and the 21st (C, F) of October 2011 at littoral and central stations.

abundance, vertical and inter-stations distributions of sporangia and prevalence of infection were detailed only for *R. akinetum*.

Independently of the considered sampled station, *R. akinetum* presented an important and significant increase ($p<0.018$) for both abundances of sporangia and prevalence of infection of akinetes during the sampling period (Fig.4 A-F). In addition, these two parameters presented no significant difference between the two stations. Even if some sporadic significant differences were reported (Pr_{8m} and Pr_{2m} were lower than others depths the 7th and 14th of October, respectively; Pr_{2m} was higher than others depths the 21st of October), the prevalence of infection of akinetes was quite homogeneous all along the water column for each date (Fig.4 D-E). Additionally, the prevalence of infection increased significantly during all the sampled period. It presented an average value of 4.7% ±1.2 on the 7th of October at the center, and then showed a 2.8 fold higher value on the 14th of October, to finally reach an average value of 31.9%±3.2 of akinetes on the 21st of October at the central station. Concerning the intensity of infection, no difference was reported between central and littoral site for all dates. Moreover, this parameter did not differ between the first two dates but was significantly higher the last date, with an average value of 1.4±0.06 at the central site.

The dynamics of sporangia was closely linked to the prevalence and the intensity of infection and to the host density, with a significant correlation found with the abundance of akinetes ($rs=0.97$, $p<0.001$) (Fig.5). As the prevalence of infection of akinetes is quite similar all along the water column, whatever station and date, *R. akinetum* abundances were quite similar to the dynamic of its host (*R.akinetum* abundances were higher in the upper water layers). This chytrid species abundance globally increased during the sampling period. Interestingly, the increase of the abundance of sporangia was higher during the first seven days than after. Indeed, this increase was at approximately 30 fold between the 7th and the 14th of October, but at only 5 fold between the last two dates (Fig. 4A,B and Fig. 5). The maximum sporangium abundance ($1.8\pm0.14\times10^5$sporangia.l^{-1}) was recorded the 21st of October at 2m. The same increase was observed at the littoral site, where the maximum value ($2.08\pm0.12\times10^5$sporangia.l^{-1}) was also reported at 2m the 21st of October.

During the sampling period, each stage of *R. akinetum* life cycle was observed. The comparison of their relative abundances did not reveal any significant differences between sampled depths, nor between the sampled stations (Fig. 4A-C). Nevertheless, temporal differences appeared. On

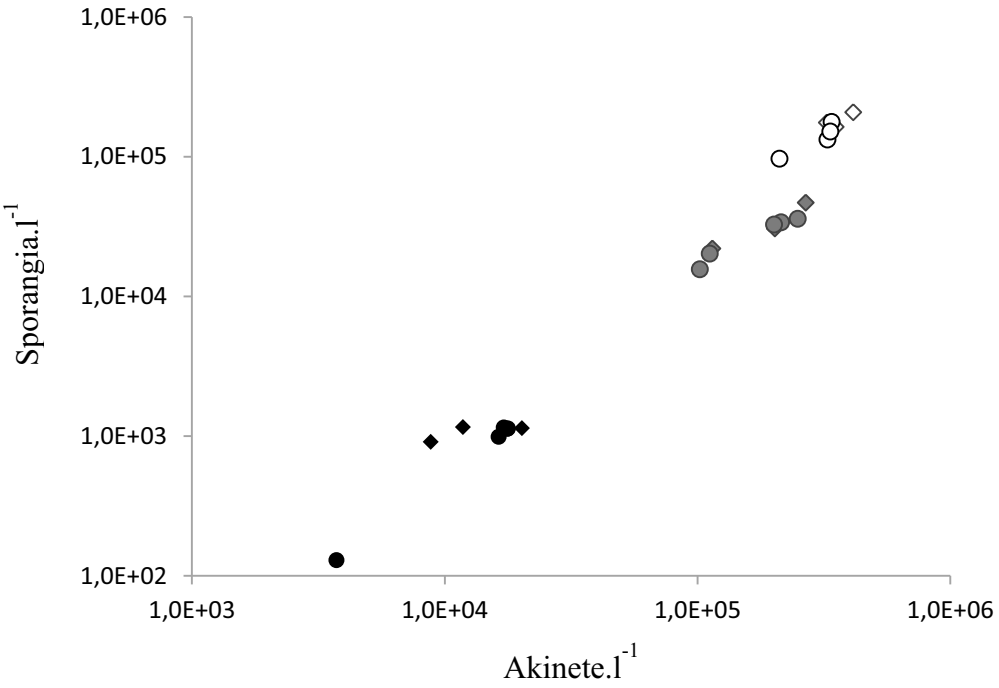


Figure 5: Relationships between sporangia and akinete abundances at littoral (circle) and central stations (diamond) established for the 7th (black), 14th (grey) and 21st (white) of October 2011.

7th October, *R. akinetum* presented a majority of sporangia involved in the maturation phase. The 7th of October, the biovolume of mature and empty sporangia averaged 1749.8±618.1µm^3 and did not differ compared to the value which was observed at the littoral station (1647.7±594.9µm^3). By using the conversion factor (CF=0.0172 zoosp.µm^3) we established a theoretical capacity of each sporangium to produce an average of 30.01 zoospores, which did not significantly differ to the experimental values (28±5 zoosp. per spor.) derived from our direct microscopic observations. The 14th of October, the young stages of *R. akinetum* dominated the *R. akinetum* population (Fig. 4B). At this date, mature and empty sporangia presented an average value of 1513.6±365.1µm^3 which was equivalent to a theoretical production of 26.03 zoospores per sporangium. Even if both of these average values were lower compared to the previous sampling date, they did not significantly differ to each other. Finally, the 21st of October presented an equal distribution of sporangia between young and maturation phases, and average biovolumes of sporangia presented no significant differences compared to the earlier sampling dates. The relative abundance of empty mature sporangia was always lower compared to other phase, for all sampling dates, stations and depths (Fig. 4 A-C).

Discussion

The aim of this study was to investigate the spatiotemporal variations of specific host-parasite pairing involved in the cyanobacterium-chytrid systems *Anabaena macrospora* and *Rhizosiphon spp*. In this study, we coupled temporal variations to vertical and horizontal distributions by comparing five different depths and two contrasted sampling stations in the same eutrophic lake. To the best of our knowledge no study has investigated such type of combined variations at the natural community level of host-chytrid ecology.

A stratified host community. The cyanobacterial host community was exclusively composed of *Anabaena macrospora*. This cyanobacterium is able to regulate its buoyancy during thermal stratification (Oliver*, et al.*, 2012) and tends to form surface bloom which can be wind-driven and distributed horizontally (Pobel*, et al.*, 2011). In our case study, vegetative cells presented both vertical and horizontal variations, especially when the bloom peaked up (October 14th). In parallel, we underlined a vertical stratification of akinetes. It seems that their production occurred in the upper water layers, as the significantly lowest density was reported in the deepest depths and the ratio akinete density/ vegetative cell density was significantly higher at 0.5m (e.g. 1.05±0.2×10^{-3} at 0.5m vs 7.35±0.5×10^{-4} at 2m the 7th of October), whatever the sampling date and site. Under laboratory conditions it was reported that diverse abiotic

conditions such as the depletion of phosphate or the decrease in temperature could be responsible to the akinete differentiation (Sutherland*, et al.*, 1979, Fay*, et al.*, 1984, Li*, et al.*, 1997). However, during our sampling period, the temperature was homogenous in the epilimnion and the 6 first meters of the lake was well mixed, leading to a quite homogenous nutrients concentration that could not explain our significant stratification in akinete production. Nevertheless a recent study showed that light intensity could be a driving force of akinete differentiation (Moore*, et al.*, 2005). Authors revealed that an increase of 4 fold in light intensity resulted in a 14 fold increase in the abundance of akinetes for *Cylindrospermopsis raciborskii*. This could explain why the akinetes seem to be produced at the upper layer of the water column and why their abundance decreased with depth during a major part of our sampling period.

An homogenous spatial distribution of chytrid infection. We reported two chytrid species parasitizing *Anabaena macrospora*. Despite these parasites belong to the same genus *Rhizosiphon*, they differ both by their type of host cells and by their abundances. Actually, *R. akinetum* presented a maximal abundance more than 11 fold higher than that observed for *R. crassum*. Takano *et al.,* (2008) reported similar differences between two chytrid species parasitizing two different type of host cells (heterocystes and akinetes) of the cyanobacterium *A. smithii*. As all critical parameters were not available for *R. crassum* (such as all life stages), we focused the spatiotemporal variations of chytrid infection on the mature akinete - *R. akinetum* pairing. During this study, we did not report any relationships between vertical nor horizontal host distributions and associated infection parameters (prevalence and intensity of infection). For each date and whatever the host density was, the fungal infection was homogenous within the Lake Aydat (Fig. 4D-E). It means that the sensitive host population did not find any water layer refuge face to parasitism, even when the lake was stratified. This has been suggested by Doggett & Porter (1996) for the diatom host *Synedra accus*. Additionally, it was reported that some fungi exhibited phototaxis (Jékely, 2009), implying that infection parameters should be higher in the upper water layers. However, we were not able to observe any significant difference in the vertical distribution of these parameters, including those of the 6 life stages. This suggests that phototaxis was a minor behavior for the distribution of *R. akinetum* population. Moreover, because we also observed that the severity of chytrid infection presented no vertical or horizontal difference, we consider that susceptible and resistant sub-populations of cyanobacteria were homogeneously distributed within the lake.

Temporal variations of chytrid infectivity: influence of environmental factors. By taking under consideration the time scale, it clearly appeared that fungal infection was closely linked to the host density (r_s=0.97, $p<0.001$). This density dependence of chytrid infection could be due to the fact that *R. akinetum* and its host are equally sensible to the same range of abiotic factors, as it was previously demonstrated for the chytrid-diatom couple *Rhizophidium planktonicum-Asterionella Formosa* (Bruning, 1991). In our study, *Rhizosiphon akinetum* and akinete abundances presented the same dynamics: important increases during the first week followed by minor ones. The first important rise of *R akinetum* can be explained through the life cycle duration and the success of infection. During the one week duration between two sampling dates, we can expected the achievement of at least one whole life cycle, based on the life cycle duration that we recently estimated at 3 days for of *R crassum* (Gerphagnon *et al.* 2013). On the first day of our sampling (7th of October), the stage 3, corresponding to the first stage of the maturation phase, dominated (50±1%) the chytrid population which presented at the end of their maturation phase a theoretical zoosporic content of 30±9 zoospores per sporangium. These zoospores were released and started to infect akinetes before our second sampling point (14th of October) as it was demonstrated by the domination of young stages among the population of *R. akinetum* for this second date (Fig. 4B). During this period, we observed a high success of chytrid infection, illustrated by the 30 fold increase in the abundance of sporangia, which was in the same range than the zoosporic content. These results suggested that almost all zoospores released in their environment caused an infection within the following week.

In contrast, although the theoretical zoosporic content did not significantly differ between October 7th (30±9 zoosp.spor^{-1}) and October 14th (26±6 zoosp.spor^{-1}), it appeared that only one over the six zoospores released the 14th of October has been responsible for a successful infection on the 21st of October. This important difference reported about zoospore infectivity could be explain by *i)* a decrease of infectivity of zoospores or *ii)* a loss of zoospores due to grazing. By impacting the sporangia maturation time, zoospore infective lifetime and the number of zoospore per sporangium, temperature was probably the most important abiotic factor driving the chytrid parasitism (Bruning, 1991, Piotrowski*, et al.*, 2004). During the first week of our sampling period, the temperature of epilimnion did not vary significantly (-1°C), whereas the last week was characterized by a sudden drop in water temperature (-3°). So, the decrease of temperature could impact the infective life time of the zoospores of *R. akinetum*, which consequently have not been able to infect a novel akinete few days later. In the same way, it was shown that the infective life time of the zoospores of

Rhizophidium planktonicum, a parasite of the diatom *Asterionella formosa*, could also be reduced by changes in temperature, especially an increase of 4°C of temperature (Bruning, 1991). In addition, diatoms are known to present lower optimal temperatures than cyanobacteria (Paerl & Huisman, 2009). We may thus hypothesize that lifecycle traits of parasitic chytrids vary according to their hosts. This suggestion is supported by a previous study on *Rhizophidium sphaerocarpum-Spyrogira sp*. pairing, where authors reported that optimal conditions for chytrid infection of the cyanobacterium *Spyrogira sp*. was 30°C (Barr & Hickman, 1967) which was largely higher than that it was reported for *R. planckonicum* (Bruning, 1991).

A loss of zoospores can also be due to grazing as it was demonstrated by Kagami *et al.,* (2011) and Kagami *et al.*,(2007) who have shown that *Daphnia* and copepods could graze actively on zoospores. Moreover, Kagami *et al.,* (2004) have reported that the grazing pressure exerted by *Daphnia* could significantly reduce the intensity of chytrid infection on the diatoms. As our last sampling week was marked by the decline in the cyanobacterial bloom, corresponding usually to an increase of zooplankton community (Christoffersen*, et al.*, 1990), we cannot reject the hypothesis of a massive grazing loss of zoospores between the 14th and the 21st of October.

Ecological implications of fungal infection on cyanobacterial population. During our sampling period, akinetes were highly infected, with 36.6 ±2% being parasitized the 21st of October. These cells constitute the resting spores of *Anabaena macrospora*. They are the only cells which overcome unfavorable conditions and lead to the colonization of the water column by cyanobacteria when favorable seasonal conditions for growth return (Baker & Bellifemine, 2000). Kravchuk *et al.,* (2011) underlined that the size of the inoculum (akinete density in sediment) is one biotic factor which determines the proportion of *Anabaena flos aquae* in the total phytoplankton biomass. Additionnally, Tsujimura *et al.,* (2000) have suggested that the start of bloom formation was related to the quantity of *Microcystis sp.* colonies in the sediment. By impacting akinetes, fungal parasitism could be responsible for an important loss of the inoculum size. Consequently, chytrids could delay or be responsible of a decrease in the competitive ability of their next years' cyanobacterium host populations.

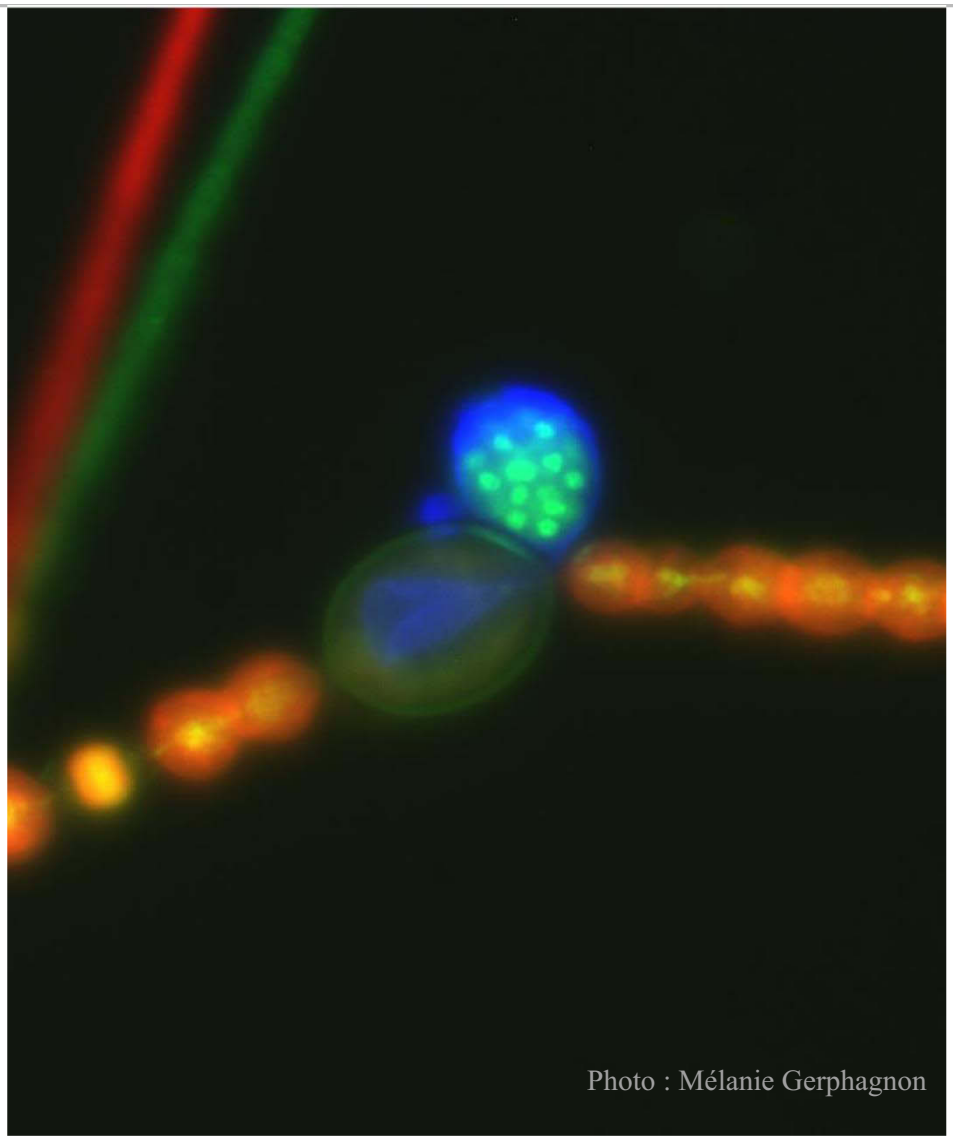
In addition, by infecting akinetes, chytrids could severly affect the genetic structure of cyanobacterial populations from year to year. Indeed, as akinetes are differentiatied from a clonal vegetative cell, their genetic structure reflect the genetic structure of the cyanobacterial

bloom and they constitute the "genetic seed bank" of cyanobacterial population. Because the genetic structure of host population is a major factor conditionning its susceptibility/resistance against chytrid infection (De Bruin*, et al.*, 2004), the parasitism of this "genetic seed bank" may have strong impact on the next years' cyanobacterial population responses to chytrid infection.

In conclusion, our investigation revealed that even if the host population was spatially structured, the fungal infection was homogenous within the lake. Additionally, the use of the double staining method suggested that lifecycle traits, such as effectiveness of infectivity of parasitic chytrid of cyanobacteria could be impacted by the same range of temperature and mixing conditions than its host. The high level of akinetes infection observed in our study also suggests that chytrid parasitism on akinetes may have sizeable consequences on host genetic structure and host proliferation from year to year. Clearly, further investigations of cyanobacteria-chytrid pairings under controlled conditions are required to confirme such relationships. Finally, the use of a spatiotemporal survey including vertical and horizontal scale variations of the fungal parasitism has highlighted that the chytrid infection seems to be forced more by the host dynamic than by the spatial distribution. Therefore, in order to improve the knowledge on host-parasite interactions under natural conditions, we suggest focusing the sampling strategy on temporal rather than on spatial scales.

Chapitre 4

Relation hôte-parasite : focus sur les paramètres influençant la fécondité des chytrides



Préambule

Les chytrides possèdent deux phases distinctes dans leur cycle de vie, une phase parasitaire, les sporanges, et une phase de dissémination, les zoospores (Sparrow, 1960, Ibelings*, et al.*, 2004). Les études menées sur les interactions phytoplancton-chytrides se sont, pour la plupart, focalisées sur l'impact du parasitisme sur les communautés hôtes (Sen, 1987, Kudoh & Tokahashi, 1990, Kagami*, et al.*, 2012) et de ce fait, sur la phase parasitaire. Les investigations menées par Kagami *et al.,* (2007) sur l'importance des zoospores comme lien entre la matière phytoplanctonique « *inedible »* et les niveaux trophiques supérieurs (CF Synthèse bibliographique) ont révélé l'importance putative des zoospores comme source de carbone dans les écosystèmes aquatiques (reviewer par Gleason & Lilje, 2009). La quantification précise et directe de la capacité de production de ces zoospores, ou fécondité, des chytrides est un paramètre important tant dans l'amélioration de la connaissance sur leur cycle de vie, que dans l'apport de précision dans les modèles tendant à expliquer les flux de de matière et d'énergie dans les écosystèmes aquatiques. Cependant, l'inexistence de méthodes simples, rapides, peu coûteuses et non restrictives à un ordre, genre ou espèce, expliquerait qu'aucune étude n'ait étudié cette fécondité chytridienne en conditions naturelles.

Pour pallier à ce manque nous avons mis au point une méthode basée sur des fluorochromes nous permettant de mettre en exergue les relations reliant la fécondité des chytrides et divers paramètres d'infection fongique, ainsi que l'influence de l'hôte sur le parasite dans le milieu naturel lors d'un bloom d'*Anabaena macrospora*. Les résultats sont présentés dans l'**Article 5** et les **résultats complémentaires** de ce chapitre.

Article 5 :

A double staining method using SYTOX-green and Calcofluor White for studying fungal parasites of phytoplankton

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Abstract

We propose a double staining method based on the combination of two fluorochromes, Calcofluor White (CFW, specific chitinous fluorochrome) and SYTOX-green (nucleic acid stain), coupled to epifluorescence microscopy for counting, identifying, and investigating the fecundity of parasitic fungi of phytoplankton and the putative relationships established between the host and their chytrid parasites. The method was applied to freshwater samples collected over two successive years during the terminal period of autumnal cyanobacterial blooms in a eutrophic lake. The study focused on the uncultured host-parasite couple *Anabaena macrospora* (cyanobacterium) and *Rhizosiphon akinetum* (Chytridiomycota). Our results showed that up to 36.6% of cyanobacterial akinetes could be parasitized by fungi. Simultaneously, we directly investigated the zoosporic content inside the sporangia, and found that both the host size and intensity of infection conditioned the final size and hence fecundity of the chytrids. We found that relationships linking host size, final parasite size and chytrid fecundity were conserved from year to year, and seemed to be host-chytrid couple specific. We concluded that our double staining method was a valid procedure for improving our knowledge of uncultured freshwater phytoplankton-chytrid couples, and so of the quantitative ecology of chytrids in freshwater ecosystems.

Introduction

Many phytoplankton species are susceptible to fungal parasitism. In freshwater lakes, the main parasitic zoosporic fungi belong to Chytridiomycota (i.e. chytrids). Recent advances in molecular biology have revealed unsuspected fungal diversity in the small size fraction (0.6-5µm) (Lefèvre*, et al.*, 2008), which comprises the dissemination form (i.e. zoospores). Microscopic studies of the reproductive stages of chytrids (i.e. the sporangium), have also revealed the wide diversity of chytrid species in the pelagic zone occurring in lakes throughout the year (Rasconi*, et al.*, 2012). Chytrids, like some other parasites, are completely dependent on their host for their nourishment and their development, which results in the death of their host (Ibelings*, et al.*, 2004). Previous studies focused on the impact of fungal parasitism on phytoplankton dynamics and have highlighted the fact that fungal parasitism has had a real impact on the decline of several eukaryotic phytoplankton species, showing that fungal parasitism is potentially implicated in the phytoplankton succession (Canter & Jaworski, 1979, Beakes*, et al.*, 1988, Van Donk, 1989, Bruning, 1991, Shin*, et al.*, 2001, Kagami*, et al.*, 2007, Grami*, et al.*, 2011).

The prevalence and intensity of infection constitute the two parameters classically used to estimate the ecological impacts of fungal parasitism on phytoplankton (Bush*, et al.*, 1997, Rasconi*, et al.*, 2009). However, it has been recognized that zoospores are an important source of carbon in freshwater ecosystems, and act as a real link between inedible host algae and higher trophic levels (Kagami*, et al.*, 2007, Grami*, et al.*, 2011). It is therefore surprising to find that the accurate quantification of chytrid fecundity (i.e. number of zoospores per sporangium), a key parameter in chytrid life cycles, is often omitted in field studies. As far as we are aware, no study has directly investigated the classical parameters of fungal infectivity alongside the abundance of zoospores, or identified any host parameters that could influence them. Nowadays, a precise investigation of these questions requires chytrid cultures (Bruning, 1991). However in freshwater lakes, the phylum of Chytridiomycota is dominated by uncultured fungi (Monchy*, et al.*, 2011, Jobard*, et al.*, 2012). This could explain why the effects of various different parameters (light, temperature, and nutrients) on chytrid development have only been investigated on a single host-chytrid couple *Asterionella formosa-Rhizophidium planktonicum* (Bruning & Ringelberg, 1987, Bruning, 1991, Bruning, 1991). Moreover, to the best of our knowledge, the survey of the putative relationships between host cell size and chytrid fecundity during a field study has only been mentioned once and even then not directly demonstrated for the couple: *Stephanodiscus alpinus*-

Zygorhizidium sp. (Holfeld, 2000). To overcome the methodological limitations, we propose a double staining method based on a combination of two fluorochromes (Calcofluor white (CFW) and SYTOX-green) coupled with epifluorescence microscopy to identify, count, and investigate the interactions that exist within uncultured host-parasite couples. We applied our method to the uncultured couple formed by the cyanobacterium *Anabaena macrospora* and one of its chytrid parasites*, Rhizosiphon crassum*, collected during a field study. We also investigated the prevalence and intensity of infection, host cell size, fungal size, chytrid fecundity, and the putative relationships connecting these parameters in this uncultured host-parasite couple.

Materials and methods

Study site and sample collection.

Samples were collected in Lake Aydat (45°39'48''N, 002°59'04''E), a small eutrophic lake (Zmax = 15 m, surface area = 60 ha) with a large catchment area (3 x 10^4 ha), which is located in the French Massif Central region. Lake Aydat was sampled on October 18, 2010 and October 21, 2011. These sampling periods in two successive years corresponded to the termination of *Anabaena macrospora* bloom events (shown by vertical profiles obtained with a spectrofluorometric probe (BBE FluoroProbe, Moldaenke, Germany)) at what is known to be a time of peak chytrid infection (Paterson, 1960). During both samplings, the sample point was located in the center of the lake, at the maximum depth. Twenty liters of each water layer were sampled using an 8-L Van Dorn bottle. In October 2010, samples were collected in the surface euphotic layer (estimated by Secchi-disk) at 0.5m. In order to investigate vertical distribution of chytrid infection, four different depths located in both the euphotic (0.5m and 2m) and aphotic (6m and 8m) layers were sampled on October 21, 2011. To eliminate the metazoan zooplankton, immediately after being collected the samples were prefiltered through a 150 µm-pore-size nylon filter, poured into clean transparent recipients, and then transferred to the laboratory for processing. The >150-µm fraction was checked to make sure that it did not contain any cyanobacterium.

Back in the laboratory, samples were treated *i)* to study the host community (triplicate180-ml aliquots of the raw samples were fixed with Lugol's iodine) and *ii)* to investigate the prevalence and intensity of infection and chytrid fecundity using the size-fractionated community method developed by Rasconi *et al.* (2009). Briefly, 18L of the sampled water was concentrated on a 25-µm pore size nylon filter. Large phytoplankton cells

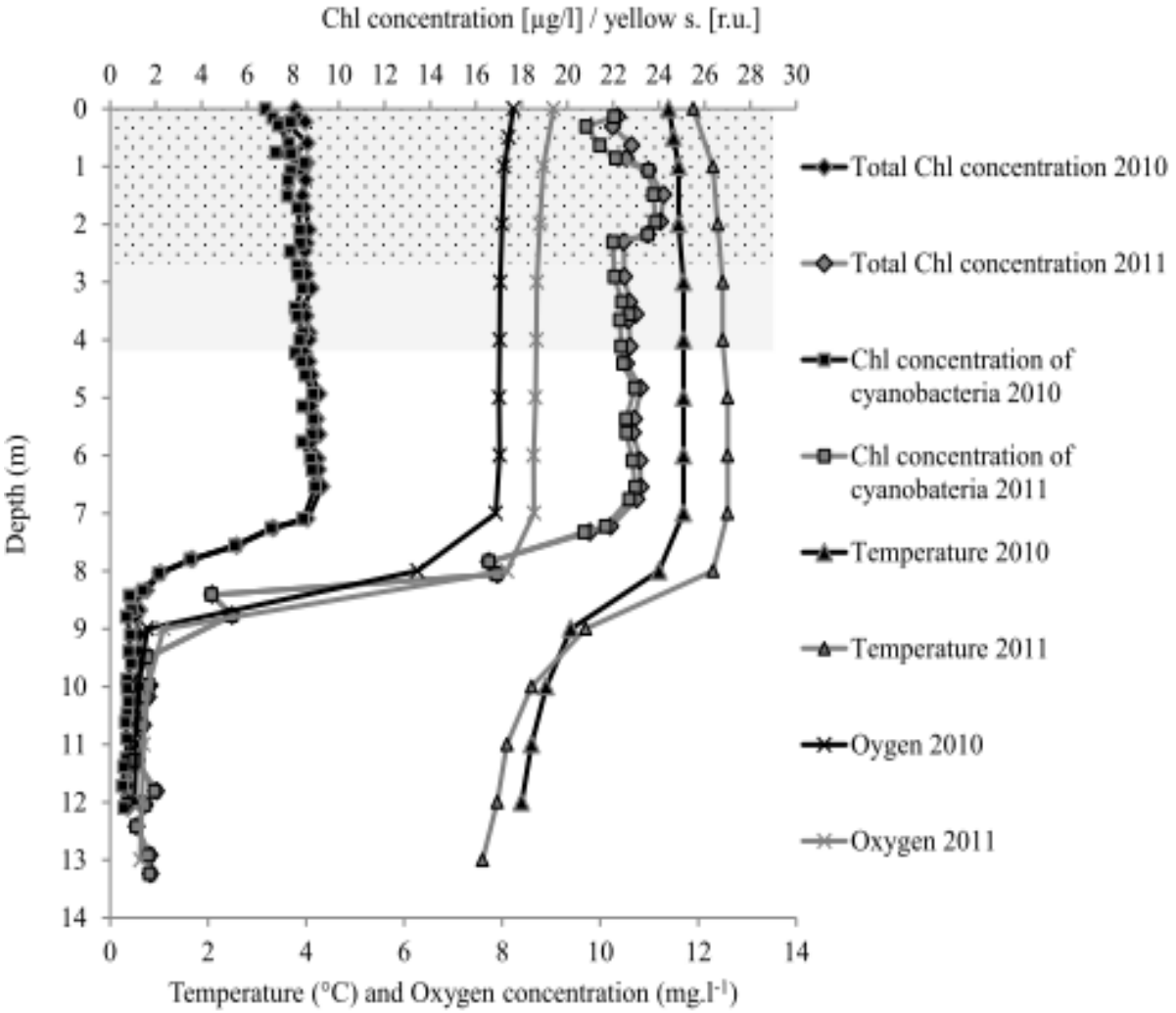

Fig S1: Temperature Oxygen and Chlorophyll concentration profiles (from total phytoplankton and cyanobacteria) for 2010 and 2011 from the center of lake Aydat. The euphotic layers were reported for 2010 (grey) and 2011 (grey with black circles)

(≥ 25µm), including the filamentous cyanobacterium *A. macrospora*, were collected by washing the filter with 0.2 µm-pore-size-filtered lake water, fixed with formaldehyde (2% final concentration), and used to carry out our double staining method described below.

Samples collected in 2011 were used *i)* to optimize our method and *ii)* at the same time to investigate classical infectivity parameters (prevalence and intensity of infection) and putative relationships between host cell size, final parasite size, and fungal zoosporic content inside a single host-parasite couple described phenotypically as *Anabaena macrospora-Rhizosiphon akinetum*. We used samples collected and fixed in October 2010 to check our method on samples that had been conserved for a year in formaldehyde and find out whether the relationships found in 2011 were maintained from year to year.

Physico-chemical parameters

Water transparency was measured *in situ* using a Secchi-disk (Z_s) and the depth of the euphotic zone (Z_{eu}) was calculated according to Reynolds (1984): $Z_{eu}=1.7\times Z_s$. Temperature and dissolved oxygen profiles were obtained using a multiparametric probe ProOdO^{TM} (Ysi, Germany). A vertical pigment profile was obtained by using a BBE Fluoroprobe® (Moldaenke, Germany) (see Fig. S1 in the Supplemental Material).

Host community analysis

Just one chytrid species, identified phenotypically as *R. akinetum*, was reported in two successive years on akinetes. As a consequence, the host community analysis was focused on the host of this chytrid: akinetes of *A. macrospora***.** Triplicate 180-ml aliquots of raw samples were fixed with Lugol's iodine. For each replicate, 5 to 20 ml (depending on the phytoplankton density) were allowed to settle overnight in a counting chamber. The cells were then counted under an epifluorescence microscope (Zeiss Axiovert 200M) following the classical Utermöhl method. The entire counting chamber was inspected, and *A. macrospora* akinetes were quantitatively analyzed by counting the numbers of immature and mature akinetes. Distinguishing between these two classes is based on their morphology (the presence of an outer envelope layer is characteristic of mature akinetes), their shape (mature akinetes are ovoid whereas immature akinetes are spherical) (Sutherland*, et al.*, 1979), and their size (16-23µm width and 21-28µm length for the ovoid mature akinetes *vs* 13-17µm diameter for spherical immature akinetes).

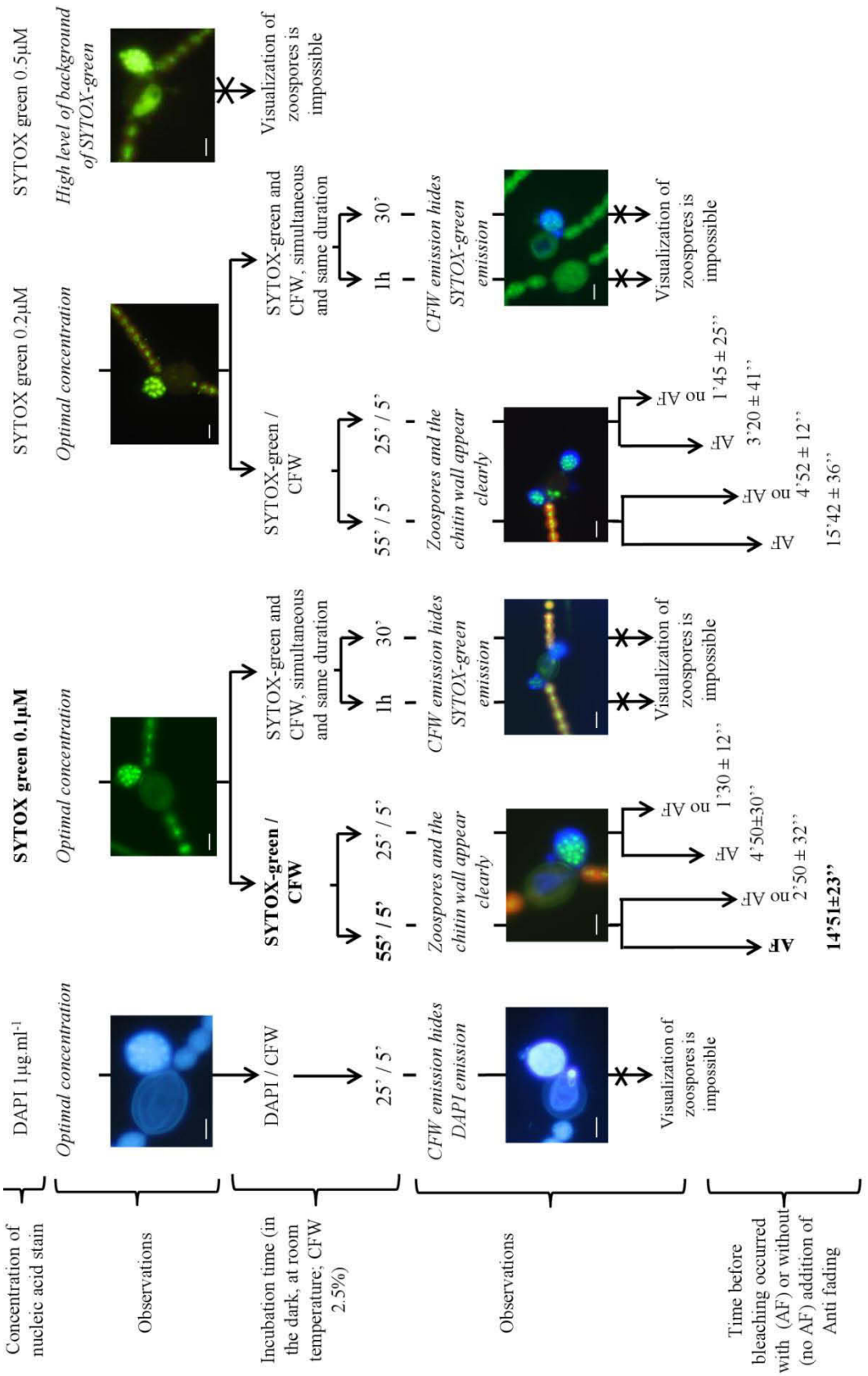

Figure 1 : Different concentration and incubation time procedures tested for double staining method and epifluorescence microscopy observation of zoosporic content and sporangia of phytoplankton parasitic chytrid. In bold: Optimal procedure. Scale bar: 10µm

Double staining of fungal parasites

Because it was impossible to investigate the number of zoospores inside sporangia of chytrids without staining, as the zoospores are simply invisible (Fig. 2A, E), we tested a double staining method combining two fluorescent stains to quantify the zoosporic content of sporangia together with the classical infectivity parameters on the same sample. Firstly, we tested the combination of the classical nucleic acid stain DAPI (4',6'-diamidino-2-phenylindole) (1µg.ml^{-1}) with the specific chitinous fluorochrome Calcofluor White (CFW, C40H44N12O10S2, Sigma catalog no F3543), (final concentration 2.5% (vol/vol) as in the original protocol (Rasconi*, et al.*, 2009) Secondly, we combined the nucleic acid stain, SYTOX-green (Molecular Probes, Invitrogen) and the CFW. A range of concentrations (0.1µM, 0.2µM, and 0.5µM) and incubation times (30 min or 1h) of SYTOX-green were tested. CFW was used at final concentration 2.5% (vol/vol). CFW was added to the samples, either at the same time as SYTOX-green (i.e. 30min or 1h of incubation) or just 5 minutes before the observations. After staining, the samples were incubated in a dark at room temperature. The sample processing procedure is summarized in Fig. 1. The optimal procedure consisted of an incubation time of 55 min with a 0.1µM final concentration of SYTOX-green, before adding CFW followed by a further incubation lasting 5 min.

Microscopic assays

To prevent bleaching, 5µl of an anti-fading solution (4:1 mixture of Citifluor and VectaShield; Vector Laboratories) was added before mounting the samples between glass slides and cover slips. The chitin walls stained with CFW were examined using UV excitation (405 nm) (Fig. 2B); zoospore nuclear genomic DNA stained with SYTOX-green was explored under blue light illumination (488nm) (Fig. 2C). Initially, some assays were done with a Zeiss LSM 510 Meta confocal microscope to visualize the zoosporic content inside the sporangia. Confocal microscopy has the advantage of automatically providing numerous images in the z axis (one every 0.65µm), which provided a precise investigation of the entire content of the sporangium, and so a very good picture quality (in 2D or 3D). However, the lasers used by confocal microscopy accelerated the bleaching of sample despite the addition of anti-fading agent, and this limited our ability to measure classical chytrid parameters (prevalence and intensity of infection). Moreover, this equipment is uncommon and expensive. We then applied our staining procedure to the samples and carried out the observations under an inverted epifluorescence microscope Zeiss Axiovert 200M (Fig. 2).

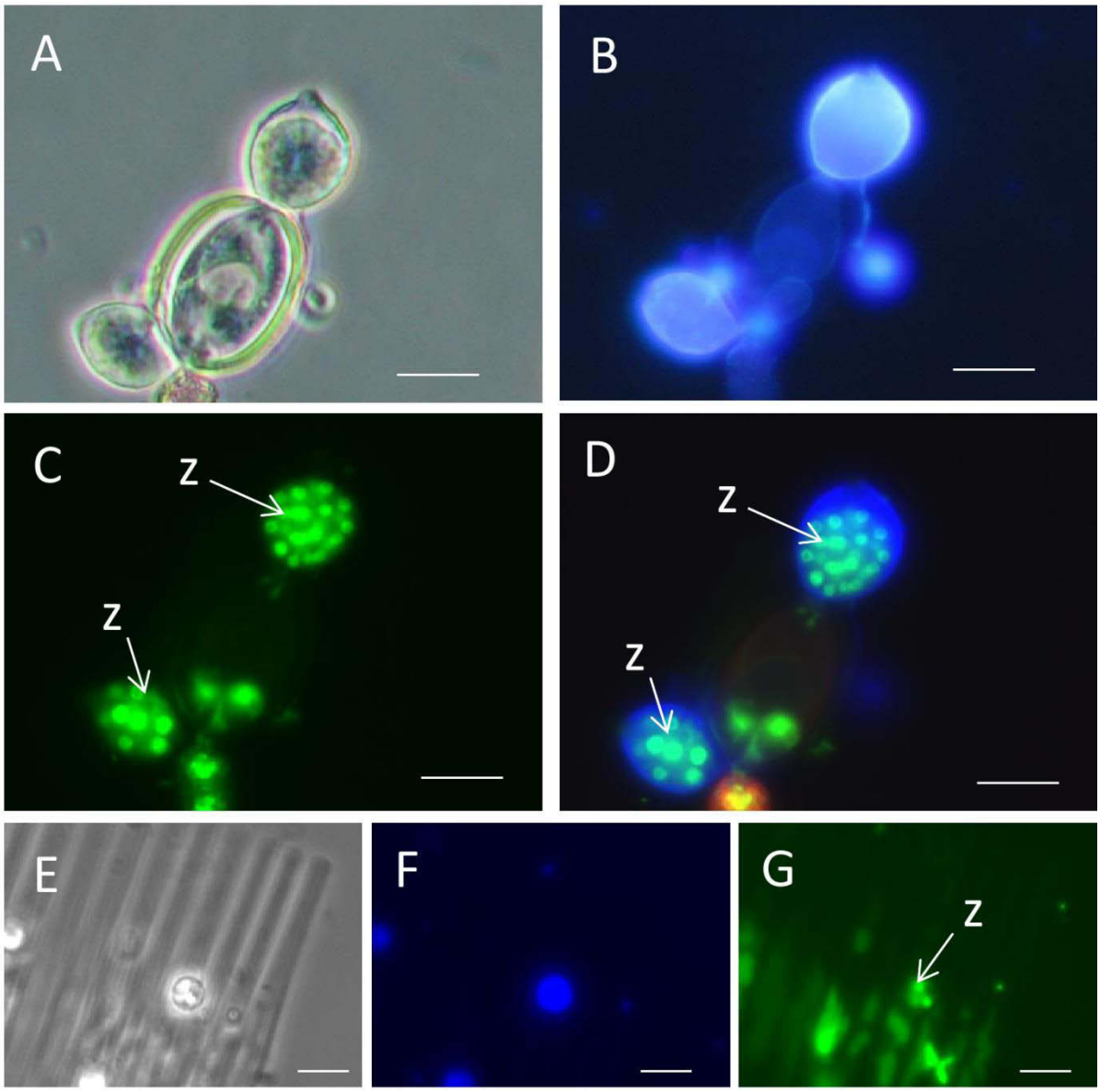

Figure 2 : Sporangium of *Rhizosiphon akinetum* (A,B,C,D) and *Rhizophidium fragilariae* (E,F,G) stained by the double staining method (CFW and SYTOX green), exited by white light (A, E), UV light (B, F), blue light (C, G) or both (D) observed by optical microscopy. Scale bar : 10µm, z: zoospores

Images of the zoosporic content were acquired at intervals of 0.8 to 1.1µm and then analyzed using Axiovision 4.1 software. Samples observed using the epifluorescence microscope (Zeiss Axiovert 200M) displayed a shorter bleaching time, which allowed us to investigate several classical fungal parameters in parallel and thus to survey the relationships between host cell size and parasite fecundity. Because no difference was observed between the relationships established between sporangium size and zoosporic content obtained using these two types of microscopy, the epifluorescence microscopy procedure was subsequently applied to all samples collected.

Chytrid parasitism

Samples were observed between glass slides and coverslips at ×400 magnification under an epifluorescence microscope (Zeiss Axiovert 200M). To confirm the target of fungal parasitism, 300 akinetes were inspected corresponding to each depth sampled, without distinguishing between mature and immature akinetes. From this count, mature akinetes were found to be the only host cells harboring *R. akinetum*, and so 300 mature akinetes were inspected for each depth. Each sample was analyzed in triplicate. Additionally, the biovolume of 100 randomly-selected, mature, infected and 100 randomly-selected, mature uninfected akinetes was calculated, assimilating mature akinetes to ovoid (Hillebrand *et al*., 1999).
Infection parameters were calculated according to the formula proposed by Bush *et al.*(1997). These parameters include the prevalence of infection of akinetes (Pr_{AK}), i.e., the proportion of individuals in a given population with one or more fixed sporangia, expressed as Pr_{AK} (%) = [(*N*i/*N*) × 100], where *N*i is the number of infected host akinetes, and *N* is the total number of host akinetes. The second parameter is the mean intensity of infection of akinetes (I_{AK}), calculated as I_{AK}= Np/Ni, where Np is the number of parasites, and Ni the number of the infected individuals within a host population. The life stage of each chytrid encountered on a cyanobacterium was noted according to Canter (1954). The first one corresponded to a zoospore, which had just penetrated the mucilage of a living host cell using a fine thread. After this encystment phase, the zoospore discharges its content into the host cell, resulting in a globose structure known as the prosporangium (stage 2). The prosporangium, expands into akinete (stage 3), followed by the emergence of an epiphytic bud corresponding to the primary prosporangium stage (stage 4), which in turn evolves into a mature spherical sporangium surmounted by a papilla (stage 5). The sixth and final stages of the life cycle of *R akinetum* correspond to the empty sporangium, after deliquescence of the papilla and extrusion of the zoospores.

For each mature and empty sporangium, the biovolume of the host akinete and the biovolume of the sporangia were calculated by assimilating sporangia to spheres and akinetes to ovoids (Hillebrand*, et al.*, 1999). Empty sporangia displaying a deformation of their cell wall were not taken into consideration in investigating relationships between host cell size and final chytrid size. For each mature stage encountered, the fecundity of the chytrid was investigated by analyzing the zoosporic content from the various different images acquired as described above. Because the abundance of mature and empty sporangia encountered during the survey of classical parameters was too low in the samples collected in 2010, 30 mature sporangia were investigated independently from these counts.

Statistical analyses

Because of unequal sample sizes, the non-parametric Kruskal-Wallis test was used, followed by a Mann-Whitney pairwise comparison with the Bonferroni correction to test differences in the biovolumes of akinetes and sporangia at different depths. Spearman's correlation coefficient was calculated to investigate relationships between the biovolume of sporangia or akinetes and the zoosporic content. The abundances of akinetes (mature or immature) were tested using a one-way ANOVA. All statistical analyses were conducted using PAST, and graphics were performed using Sigma plot 11.0.

Results

Physico-chemical parameters

The temperature and oxygen concentrations presented similar values and profiles in the two successive years. Thermoclines were located at 8m in both years, with the maximum temperature obtained in the euphotic layer ((mean ± SD) being11.7±0.3°C and 12.4±0.2°C for 2010 and 2011, respectively). The euphotic layer was well oxygenated in both these years (8.2±0.1 8.8±0.14 $mg.l^{-1}$ for 2010 and 2011, respectively) (see Fig. S1 in the supplemental material).

The host community

Vertical profiles obtained using the BBE fluoroprobe revealed the strong predominance of cyanobacteria in Lake Aydat at both sampling dates (from 72% of total concentration of chlorophyll a at 10m in Oct.2010 to more than 99.5% at 1m in Oct.2011) (see Fig. S1 in the Supplemental Material). For each year, the cyanobacterial community was heavily dominated

TABLE 1. Biological variables mesured in the eutrophic Lake Aydat the 18 Oct.2010 and the 21 October 2011. (Minimum (Min), maximum (Max) and mean (± Standart variation, SD))

	2010 (0.5m)		**2011 (0.5, 2, 6 and 8m)**					
			Min		Max			
Parameters	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
Density of mature akinete (M.A.) ($\times 10^5$ akinete.l^{-1})	3.69	0,72	2.11 [d]	0.28	3.4 [b]	0.53	3.3	0.6
Density of immature akinete (I.A.) ($\times 10^5$ akinete.l^{-1})	1.1	0.2	1.19 [d]	0.5	1.66 [c]	0,15	1.45	0.25
Prevalence of infection of *R. akinetum*	4.6	1	28.2 [a]	0.02	36.6 [b]	0.02	31.9	3
Intensity of infection of *R. akinetum*	1.2	0.1	1.42 [b]	0.03	1.46 [c]	0.1	1.44	0.02
Number of life stage 1 of *R. akinetum*	3.7	0.5	21.3 [a]	6	34 [b]	7.8	29.58	7.0 8
Number of life stage 2 of *R. akinetum*	2.3	1.1	40.3 [d]	1.15	45 [a]	1.5	42.41	11.8
Number of life stage 3 of *R. akinetum*	1	1	37 [a]	10	45.6 [b]	9.2	40.5	8.25
Number of life stage 4 of *R. akinetum*	0,3	0,6	1.6 [a]	0.5	2 [d]	1	2.25	1.35
Number of life stage 5 of *R. akinetum*	2,6	0,5	4 [c]	3	9.6 [b]	3.2	6	3.2
Number of life stage 6 of *R. akinetum*	4,33	1,5	14 [a]	4.5	19.6 [b]	4.5	16.25	4.5

[a] Values reported at 0.5m
[b] Values reported at 2m
[c] Values reported at 6m
[d] Values reported at 8m

by the filamentous blue-green alga *Anabaena macrospora*. 81.6±7.2% of *A. macrospora* filaments presented either mature (M.A) or immature (I.A) akinetes. In October 2010, the density of mature akinetes (M.A) (3.69± 0.7×10^5 ak.l^{-1}) was 3 times greater than that of immature akinetes (IA) (1.1± 0.2×10^5 ak.l^{-1}) (Table 1).

In 2011, at all depths, on average the M.A density was double that of I.A ($3.4\pm0.53\times10^5$ M.A per liter *vs* $1.65\pm0.15\times10^5$ I.A per liter at 2m, for example). M.A and I.A exhibited significantly higher values ($p<0.001$) in the first three–depths than in the deepest depth. No significant difference was noted between different depths (for the biovolume of mature akinetes data not shown). Because no infection of I.A was reported during the first investigation, we subsequently focused solely on mature akinetes.

Staining of chytrids

The zoosporic content of sporangia was stained by DAPI (Fig. 1), but the combination with the CFW was unsuccessful, as the two dyes presented two rather close emission wavelengths. The best staining intensity obtained for the zoospore DNA content was recorded with concentrations of SYTOX green of 0.1 and 0.2 µM (no difference was noted between these two concentrations). In contrast, a high level of background "noise" was found at the highest concentration (0.5µM). Despite adding anti-fading agent, considerable bleaching was observed for incubation times of less than 1 h with SYTOX-green (Fig. 1). We therefore adopted an incubation time of 1h and a final SYTOX-green concentration of 0.1µM. In contrast to the nucleic acid stain, an incubation time of 5 min was long enough for CFW to stain its target, the chitin wall. However, when it was added along with SYTOX-green, the fluorescence emitted by CFW hid the clear fluorescence of zoosporic nuclear genomic DNA (Fig. 1). CFW was therefore added 5 min before the end of staining with SYTOX-green. To summarize the procedure finally adopted, samples were incubated in the dark for 55minutes with 0.1µM of SYTOX-green and then, 5min before the direct microscopic observations, the CFW was added at a concentration of 2.5% of the stock solution. We considered this procedure to provide optimum conditions for the double staining method proposed in this study.

Prevalence and intensity of infection of mature akinetes

In October 2010, 4.6±1% of akinetes were infected by *R. akinetum* and they presented a mean intensity of infection of 1.2±0.1. In the following year, infection reached 36.6% of akinetes, and no significant difference was found between depths, with the exception of Pr_{AK} at 2m (36

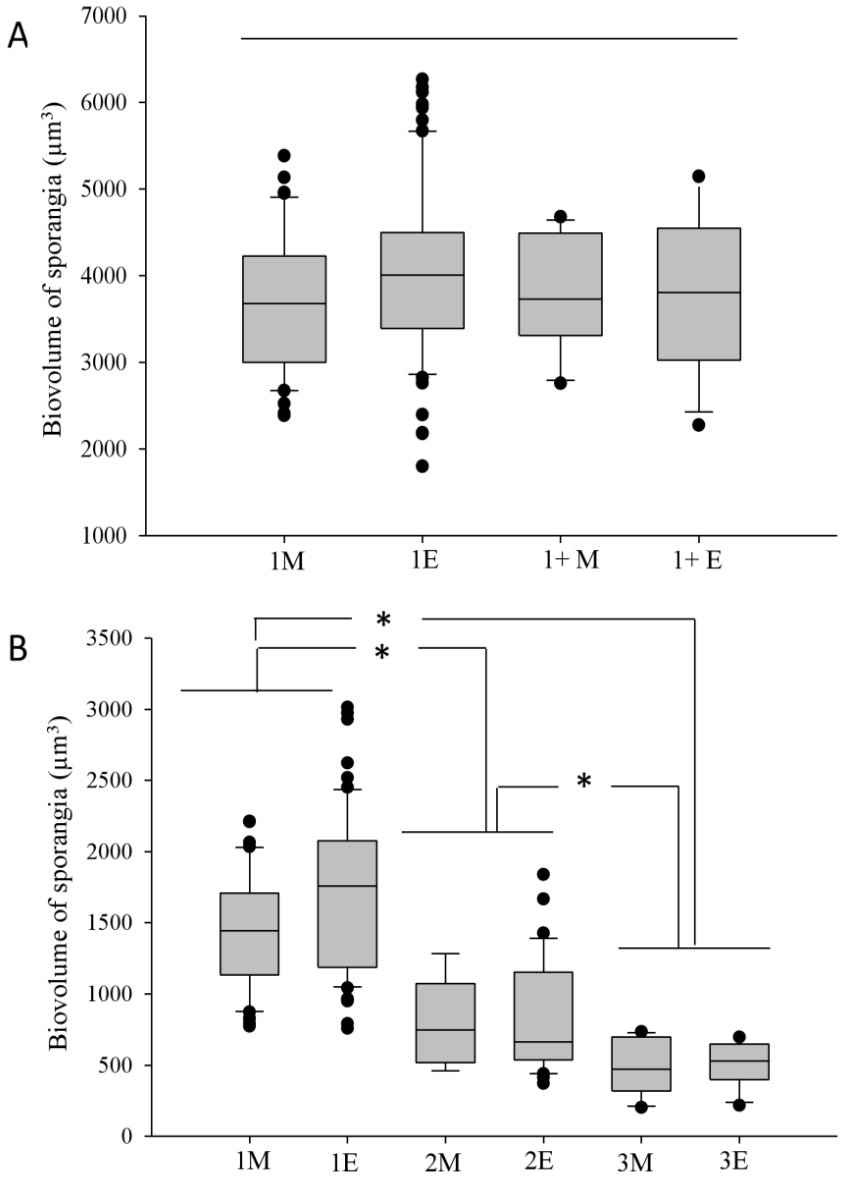

Figure 3: Biovolume of host akinetes (A) and sporangia (B). (A) Akinetes were parasitized by one (1M) or several (1+M) mature sporangia or by one (1E) or several (1+E) empty sporangia. (B) Sporangia of *R. akinetum* were mature (M) or empty (E) and were alone (1M and 1E) or there were two (2M and 2E) or three (3M and 3E) per akinete. Box plots surmounted by a black line indicate no significant difference (Mann-Whitney pairwise comparisons, $P<0.05$); asterisks indicate significant differences (Mann-Whitney pairwise comparisons, $P<0.05$). Horizontal lines represent variable medians, boxes delineate the first and the third quartiles, and circles represent potential outliners.

±2%), which was significantly higher ($p<0.05$) than Pr_{AK} at 0.5m (28±2%). The mean intensity of infection also appeared to be quite stable throughout the water column (1.44±0.02). The 6 different life stages of *R. akinetum* reported by Canter (1954) were observed during both years and in each water layer. Comparing their abundances did not revealed any significant differences between the depths (Table 1). Each stage of life presented a significantly lower abundance in 2010 than in 2011 ($p<0.001$).

Relationships between host size, fungal size, and chytrid fecundity

Because the prevalence of infection and consequently, the abundances of stage 5 (mature sporangia) and 6 (empty sporangia) were too low in 2010, the relationships between host biovolume and the final size of the sporangia was investigated for samples collected in 2011. In 2011, stage 5 and stage 6 represented 5±2% and 12±3% of the total sporangia encountered, respectively. Their abundances were quite uniform throughout the entire water column, around 6 mature sporangia and 17 empty sporangia were counted in each set of 300 akinetes (Table 1). Their individual biovolumes, ranged between 233.5 and 2213.7µm^3, and, 217.3 and 3013.09 µm^3, respectively, with no significant difference. This allowed us to investigate the relationship between the biovolumes of host akinetes and sporangia, and the zoospore contents, independently of vertical distribution.

Relationship between host biovolume and the final size of the sporangia. Individual biovolumes of parasitized and uninfected akinetes were not significantly different, with average values of 3694.07±887.8 µm^3 and 3490.08±823.1 µm^3, respectively (data not shown). Moreover, the biovolume of host akinetes did not vary significantly, whether they were parasitized by one or more fungi or depending on the fungal infection stage (stage 5 or 6) (Fig. 3A). In contrast, the biovolumes of *R. akinetum* did present a significant difference (Kruskal-Wallis test, $p<0.001$) according to the intensity of infection: when the intensity of infection increased, the sporangium size decreased significantly (Mann-Whitney pairwise comparisons, $p<0.05$) (Fig. 3B). The mean individual biovolume of the mature sporangia was reduced on average by 67.1% for mature and 71.62% for empty sporangia, when the intensity increased from 1 to 3 (Fig. 3B). Nevertheless, no difference was found regarding the biovolume of *R. akinetum* for the different life stages of the chytrids (i.e. whether they were mature or empty) for equal infection intensities (Fig. 3B). As a consequence, we pooled data from these two stages against the biovolume of their host akinetes. The results revealed a

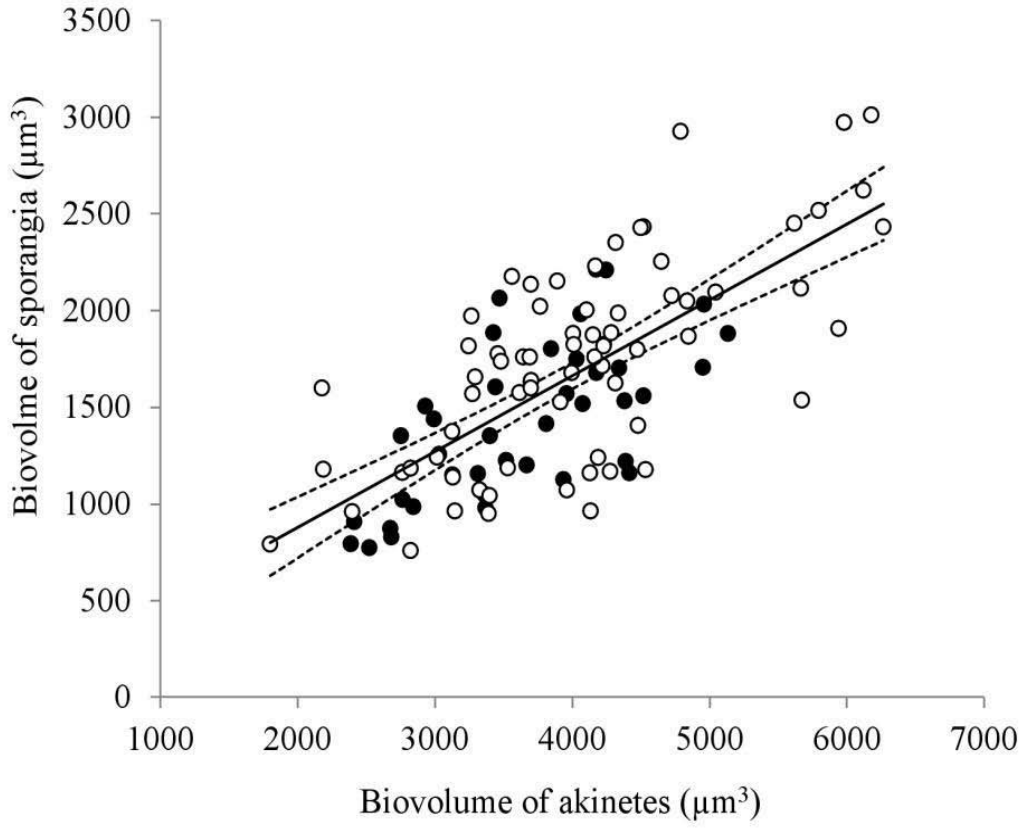

Figure 4: Biovolume of fungal parasite sporangia plotted against the volume of host akinetes. Hollow circles represent empty sporangia. Filled circles represent mature sporangia. Linear regression values and 95% confidence intervals apply to all sporangia ($y = 94.25 + 0.3923x$; $n=131$; $r= 0.79$; $P<0.001$).

significant linear relationship (y= 94.25+0.3923x; r^2=0.52; P<0.001) between the akinete size and sporangium size for an intensity of infection equal to 1 (Fig. 4).

Relationship between the sporangium biovolume and zoosporic content. Our double staining method (SYTOX-green combined with CFW) allowed us to investigate the number of zoospores in each mature sporangium. The number of zoospores per sporangium varied from 6 to 44 (Fig. 5A) and was significantly correlated (r^2=0.65; p<0.001) to the size of the sporangium (Fig. 5B). Application of our double staining method to samples collected in 2010 and preserved for one year in formaldehyde was successful. The number of zoospores per sporangium ranged from 2 to 45 and the numbers found were significantly correlated to the volume of mature sporangia (r^2=0.625; p<0.001), which did not differ from the 2011 values. From results obtained in 2010 and 2011, we established a conversion factor (CF), calculated as $CF= N_Z /V_{M.A,}$ where N_Z is the number of zoospores in the mature sporangium and $V_{M.A.}$ is the biovolume of the sporangium (μm^3). We obtained a conversion factor for *R. akinetum* of 0.0172 zoospores per μm^3 of sporangium (Fig. 5B). For the two successive years sampled, no difference was found for the conversion factor or the links established between final fungal size and their fecundity, thus we assumed that relationships established for the chytrid fecundity were conserved from year to year.

Discussion

The aim of this study was to develop a quick and easy method to explore the relationships existing between chytrid parasites and their phytoplankton host species, including parasite fecundity and the infection parameters that could influence it. To the best of our knowledge, no method has so far been devised that can be used to survey the prevalence and intensity of infection, host cell size, fungal size and zoospore production simultaneously. To fill this gap, we propose a double staining method based on a combination of two classical fluorochromes: CFW and SYTOX-green.

Previous techniques used for the direct investigation of zoospore production required culturing. Bruning (1991) assumed that the best way to quantify the zoospore content in mature sporangia accurately was to wait for the sporangium to discharge its contents, and then count how many zoospores are released. This method requires having a host-parasite couple that can be maintained under culture conditions. However in freshwater lakes, Chytridiomycota are dominated by uncultured fungi (Monchy*, et al.*, 2011, Jobard*, et al.*, 2012). Chytrids are difficult to culture and their maintenance under laboratory conditions

requires huge efforts (Gleason*, et al.*, 2007). Moreover some traits of parasites, such as zoospore production, could be altered by the culture method, which could modify the natural relationships existing between the host-parasite couple (De Bruin*, et al.*, 2008). Molecular biology method as CARD-FISH could provide quantitative data without passing by the culture condition (Amann & Fuchs, 2008). However, such method is based on the specificity of a probe with rRNA target sequences. This using requires DNA-sequences of chytrids of interest which is not always possible (as it was the case in this study). Moreover, this method is often specific to species, genus or order (Jobard*, et al.*, 2010) while the double staining method proposed in this study could be used on various species of chytrids without any discrimination. Additionally, by binding the ribosomal structures, the CARD-FISH emits a diffuse signal which could exclude a precise quantification of zoospores inside sporangia. Thus, we consider the double staining method proposed in this study to be a good candidate for avoiding the need for cultures and accurately quantify the number of zoospores per sporangium, as well as providing possible species identification of both the chytrid and the host. Calcofluor White (CFW) binds to ß 1-4 polysaccharides, such as those found in chitin, which composes the fungal cell wall (Walker*, et al.*, 1995). Rasconi *et al.,* (2009) described CFW as the best fluorochrome for studying the morphological diversity of chytrids. Because it is impossible to survey zoospore numbers inside sporangia without using a stain (Fig. 2A), and because the traditional nucleic acid stain, DAPI (4',6'-diamidino-2-phenylindole), has an emission wavelength (470 nm) rather close to that at which CFW fluoresces (435nm) (Fig. 1) (Carlisle*, et al.*, 2009), we combined the CFW with the staining nucleic acid, SYTOX green (Fig. 1, 2). This stain only binds to the nucleic acid of permeabilized cells, which is why it is commonly used to discriminate between viable and non-viable cells or spores (Green*, et al.*, 2000, Veldhuis*, et al.*, 2001). Our samples were fixed with formaldehyde (for times ranging from a few hours to one year), which is known to permeabilize cell membranes (Shapiro, 2003). SYTOX-green was not used here to identify viable cells, but was chosen for its capacity to emit green fluorescence (523nm) when it binds to nucleic acid. Under optimal conditions (incubation time of 55min with a 0.1 µM final concentration of SYTOX-green, then adding 2.5% of CFW for a further incubation time of 5min) the double staining procedure made it possible to visualize the chitin wall at the same time as the zoospore (Fig. 1, 2D), and did not interfere with identification of host cells and chytrid parasites.

Based on morphological criteria, we identified a host-parasite couple, *Anabaena macrospora-Rhizosiphon akinetum,* on October 18, 2010 and October 21, 2011. *R. akinetum*

is a well-known chytrid parasite of cyanobacteria and, more particularly, of the genus *Anabaena* (Gerphagnon*, et al.*, Canter, 1951, Sparrow, 1960, Canter, 1972). It specifically parasitizes the resting spores (i.e. akinetes) of these cyanobacteria. Not only was *R.akinetum* limited to one type of host cell, but it also seemed to be restricted to the fully grown akinetes. Whatever the abundance of *R. akinetum,* no parasite was reported on immature akinetes during the two successive years sampled, whereas mature akinetes were more or less severely parasitized (from 4±1% in 2010 to 36.6 ±2% in 2011) (Table 1). Canter (1954) described the same limitation of infection to mature akinetes in samples collected in a pond in South Bohemia. Immature and mature akinetes presented two main differences: *i)* their macromolecular composition and *ii)* their morphology. During the akinete differentiation, Sutherland *et al.,* (1979) reported a two-fold increase of total carbon and a 16-fold increase of glycogen, which constitutes the prime energy reserve of zoospores for dispersal (Suberkropp & Cantino, 1973, Gleason*, et al.*, 2008). These macromolecular changes could explain why chytrids are attracted by mature akinetes. In fact, some authors have proposed a possible chemotactic attraction between host and parasites (Mitchell & Deacon, 1986, Moss*, et al.*, 2008). Moreover, Holfeld (2000) has suggested that host size could be a driving factor of infection. This author also reported that infected cells of the diatom *Stephanodiscus alpinus* had a higher median diameter than uninfected cells. This hypothesis could explain why fungi are restricted to mature akinetes. However, in our study no difference of biovolume was reported between parasitized and uninfected mature akinetes (data not shown), and so this hypothesis could not be the only explanation. In addition, no correlation was found between host biovolume and intensity of infection. These last two observations contradict the suggestion that host size exerted any driving force on *R. akinetum* distribution (Fig. 3A).

However, the host size did seem to drive chytrid parasitism by influencing its fecundity. In fact, we pointed out a significant correlation between host cell size and the final parasite size. This phenomenon, currently observed in parasitology (Morand*, et al.*, 1996, Atkinson & Sibly, 1997, Johnson*, et al.*, 2005), has just been reported once in the context of the fungal parasitism of phytoplankton (Holfeld, 2000). Chytrids, like some other parasites, feed on the contents of their host (Ibelings*, et al.*, 2004) and so, their development and final size are closely dependent on these contents. However, host content is limited, and so as infection intensity increases, the less content is available for each individual parasite. This could explain why parasite size is significantly lower when akinetes presented multiple infections (Fig. 3B). With regard to its development, the fecundity of chytrid species was

closely related to sporangium size. During maturation, sporangia undergo multiple mitotic divisions and the cytoplasm is totally transformed into several uninucleated zoospores (Beakes*, et al.*, 1992, Berger*, et al.*, 2005). Using the double staining method proposed in this study, we have been able to identify this close relationship in *Rhizosiphon akinetum* and to establish a conversion factor (CF) of 0.0172 zoospores per μm^3 of sporangium for *Rhizosiphon akinetum.* In our study, the conversion factor (number of zoospores per μm^3 of sporangium) did not significantly differ between the different years (Fig. 5A), depths, or infection intensities. Our results are supported by experimental results reported by Bruning (1991), revealing that CF is independent of light, temperature, or intensity of infection. This established factor affords access to the mean zoospore content in sporangium, by the simple measurement of biovolume of sporangia, and the putative number of zoospores that had been released in environment, by the measurement of empty sporangia, thus avoiding the need to count numerous zoospores. The access to the capacity of zoospore production combined with the prevalence of infection may provide interesting avenues on both the effectiveness of fungal infection and the host susceptibility/resistance face to fungal parasitism.

Otherwise, the conversion factor obtained for *R. akinetum* was approximately one tenth of that reported for *Rhizophydium planktonicum* (0.166 zoospores per μm^3 of sporangium), a parasite of the diatom *Asterionella formosa* (Bruning, 1991). This marked difference could be due to an underestimation of the number of zoospores per sporangium. However, we assumed that our microscopy protocol avoided misestimating the number of zoospores. Images of the zoosporic content were acquired at 0.8 to 1.1µm intervals, which was considerably less than the diameter of the fungal zoospores of *R. akinetum* (2.5–3 µm) (Canter, 1954). Moreover, no difference in biovolume was found between mature and empty sporangia (Fig. 3B), which confirmed that we were investigating fully grown chytrid and not sporangia involved in the maturation process, which could have been resulted in an underestimation of the zoosporic content. The higher fecundity of *R. planktonicum,* in the study conducted by Bruning (Bruning, 1991), could be due to a difference in the size of zoospores. Smaller zoospores could mean that *R. planktonicum* can produce more zoospores per μm^3 of sporangium. However, Sparrow (1960) reported zoospore diameters of 3-3.7µm and 2.5-3µm for *R. planktonicum* and *R. akinetum*, respectively, which contradicts this hypothesis. Moreover, during our investigations of *R. akinetum*, we had some opportunities to observe another chytrid species, *Rhizophidium fragilariae,* a fungal parasite of the diatom

Fragilaria crotonensis (Fig. 1E, F, G). There were too few of them to permit a reliable investigation, but preliminary data revealed a conversion factor that was much closer to that

of *R. planktonicum* (0.076 zoospores per μm^3 of sporangium) and approximately 4.5 fold higher than that observed for *R. akinetum.* Kagami *et al.,* (2007) have shown that the lipid composition of zoospores is linked to the composition of its host. Diatoms are generally considered to provide better food quality than cyanobacteria (Gulati & Demott, 1997). In addition, Lord and Roberts (1986) reported that food quality could influence the zoospore production of *Lagenidium giganteum*. This hypothesis requires further investigation to confirm its, but it might signify that phytoplankton host food quality could drive the fecundity of their chytrid parasites.

In conclusion**,** we proposed a double staining method based on a combination of CFW and SYTOX-green for counting, identifying, and investigating the fecundity of phytoplankton fungal parasites and the putative relationships established between hosts and their fungal parasites. Our simple, quick, and cheap method avoids the need for culture conditions and allows direct investigations of fixed natural samples. We have established that host size does not seem to be a driving force of chytrid infectivity, but did influence chytrid fecundity. However, our conclusions are mainly based on the results of field observations. Clearly, further investigations of several chytrid species infecting different host species are required before remains necessary for accurate generalization of such relationships. Additionally, we assume that our method presents some limitations concerning the quantification of free zoospores. Molecular biology, i.e. CARD-FISH, could be a good complementary tool for chytrid species for which rDNA sequences are known (Jobard*, et al.*, 2010). Nevertheless, our method does make it possible to investigate zoospore production by chytrid species. Thus, the ability to access the production of zoospores of each chytrid species could provide interesting data for quantifying the carbon input of each species in a mixed fungal community. This could be very useful for authors trying to model the effects of parasitism in freshwater ecosystems (Bruning*, et al.*, 1992, Montagnes*, et al.*, 2008, Grami*, et al.*, 2011, Rasconi*, et al.*, 2012).

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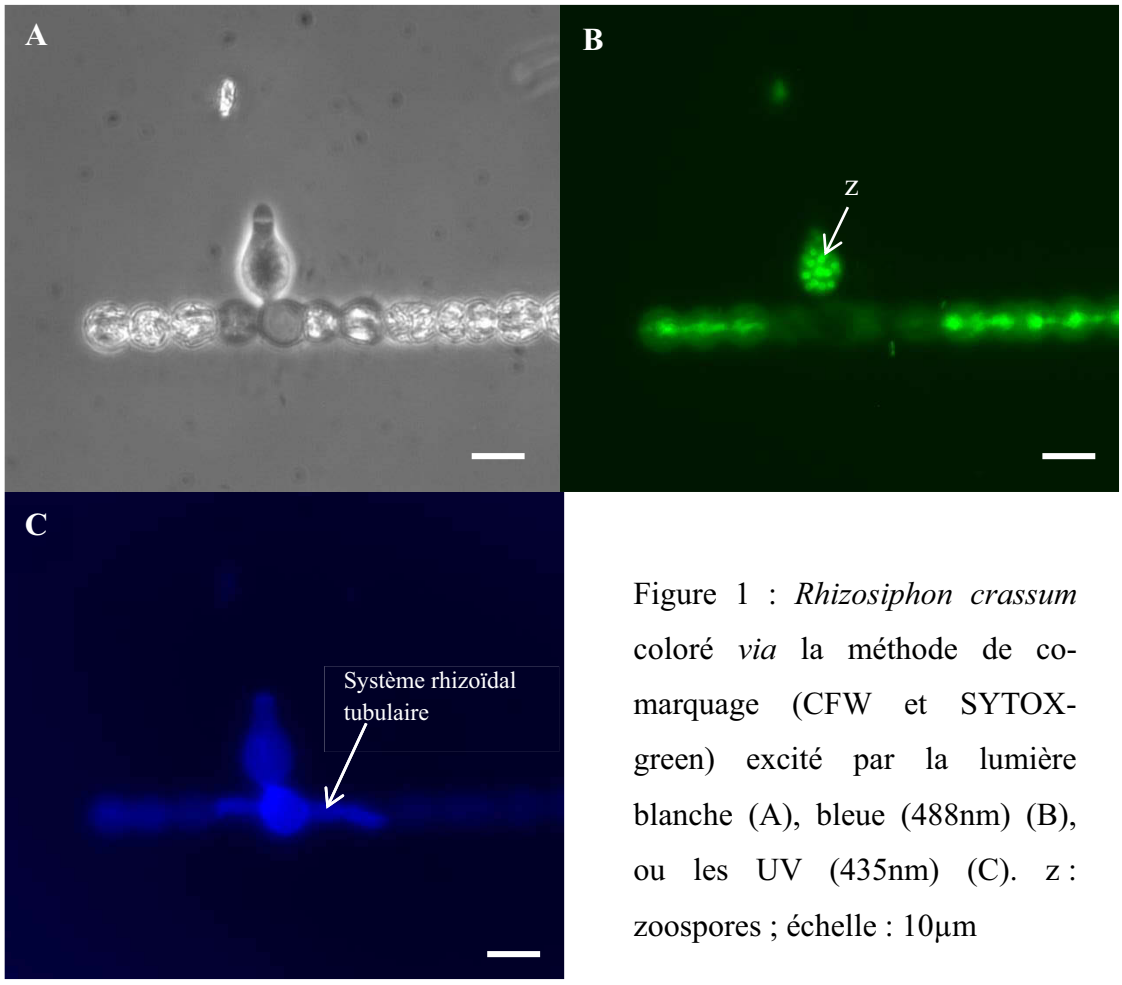

Figure 1 : *Rhizosiphon crassum* coloré *via* la méthode de co-marquage (CFW et SYTOX-green) excité par la lumière blanche (A), bleue (488nm) (B), ou les UV (435nm) (C). z : zoospores ; échelle : 10µm

Résultats complémentaires sur la fécondité des chytrides

Les résultats obtenus au cours de l'**Article 5** nous ont permis de mettre en évidence les relations existantes entre la taille de l'hôte et la fécondité du chytride *Rhizosiphon akinetum*, parasite d'*Anabaena macrospora*. De plus nous avons montré que la méthode de double marquage développée était applicable à différentes espèces de chytrides mais également que les relations établies entre la taille de l'hôte, celle du chytride et le nombre de zoospores par sporange étaient conservées quelle que soit l'année ou les conditions abiotiques (Gerphagnon*, et al.*, 2013).

Au cours des deux blooms cyanobactériens étudiés, une seconde espèce parasitant *Anabaena macrospora* a été déterminée : *Rhizosiphon crassum*. Ce champignon présente des caractéristiques de développement différentes de *R. akinetum* (développée au cours de l'étude 1 ; Gerphagnon *et al.*, 2013), notamment par le fait qu'il parasite non pas une mais plusieurs cellules hôtes grâce à son système rhizoïdal. *De facto*, nous avons i) examiné la relation entre la taille de l'hôte et la fécondité des sporanges de *Rhizosiphon crassum* et, ii) comparé la capacité de production des zoospores chez les deux espèces de chytrides parasitant la cyanobactérie *Anabaena macrospora*.

Nous avons appliqué la méthode de double-marquage combinant le CFW et le SYTOX-green (**Article 5** ; Gerphagnon *et al.,* 2013) sur *Rhizosiphon crassum* (Fig.1). Les relations entre l'hôte et la fécondité du chytride étant conservatives, des échantillons issus du bloom de 2010 (18 Oct.2010 ; 0,5m) et de 2011 (7 Oct.2011 ; 0,5m) ont été utilisés pour répondre à nos questions.

Les résultats, trop préliminaires lors de la rédaction de l'article précédent, sont présentés ci-après.

Aucune différence significative de la taille de sporange n'a pu être mise en évidence entre les deux années étudiées. Le biovolume moyen d'un sporange mature de *R. crassum* est de 588,41±241,2µm^3, ce qui est significativement inférieur à celui reporté pour *R. akinetum* (1465,3±424,2 µm^3) (Mann-Whitney pairwise comparison ; $p<0.001$). Cela corrobore le fait que le nombre moyen de zoospores produites par *R. crassum* est significativement plus faible (Mann-Whitney pairwise comparison , $p<0.05$) (20,52±7 zoospores. sporange^{-1}), comparé à celui de *R. akinetum* (26,4±8,2 zoospores. sporange^{-1}).

Le nombre de zoospores produites étant significativement très étroitement corrélé au biovolume du sporange (rs=0.79 ; p<0.0001), nous avons pu déterminer des Facteurs de Conversion (CF) pour notre seconde espèce parasitant *A. macrospora*. *R. crassum* présente un facteur de conversion plus élevé ($CF_{crassum}$=0.030 zoospores.μm^3 de sporange) que celui de *R. akinetum* ($CF_{R.akinetum}$=0.0172) (Fig. 2). Cette différence de fécondité observée pourrait être due à la taille des zoospores. Effectivement, plus les zoospores sont petites, plus la capacité de production par μm^3 de sporange est importante. Cependant Canter (1951) reporte des tailles de zoospores similaires entre ces deux espèces (2,5 à 3µm de diamètre pour *R. akinetum* et 3µm de diamètre pour *R. crasssum*). Cette hypothèse est donc à exclure.

Par ailleurs, nous avons pu montrer que le nombre de zoospores produites par *R. crassum* était lié au nombre de cellules végétatives infectées par le système rhizoïdal tubulaire (Fig. 3) (rs=0.5 ; p=0.001). Des résultats similaires ont également été observés pour *R. akinetum*. En effet nous avons pu mettre en évidence que plus l'akinète infecté présentait une taille importante, plus le biovolume du sporange était important, et par conséquent plus le nombre de zoospores produites était élevé (Gerphagnon *et al.,* 2013). Le biovolume moyen de l'hôte parasité par *R. crassum* (biovolume des cellules parasitées par le système rhizoïdal) est significativement plus faible que le biovolume moyen de l'hôte parasité par *R. akinetum* (biovolumes des akinètes infectés) (Mann-Whitney pairwise comparison ; p<0.05). Cette observation pourrait expliquer le plus faible nombre de zoospores produites par sporange. Cependant, en rapportant ce nombre de zoospores au biovolume du sporange, c'est-à-dire en étudiant la fécondité du sporange nous avons pu montrer que le taux de production de *R. crassum* était plus important que celui de *R. akinetum* ($CF_{crassum} > CF_{Akinetum}$). Le biovolume de l'hôte parasité étant plus faible, et le taux de production de zoospores plus élevé chez *R. crassum*, nous amène à considérer que, pour cette espèce, la « quantité » d'hôte parasité n'influence pas la capacité de production de zoospores.

Une seconde hypothèse pourrait être émise pour expliquer cette différence de fécondité. En effet, bien que ces deux espèces parasitent la même espèce hôte, la nature des cellules hôtes diffère. *R. akinetum* est un chytride spécifique des akinètes, contrairement *à R. crassum* qui parasite à la fois des cellules végétatives et des akinètes (Canter, 1951 ; Gerphagnon *et al,* 2013). Cependant, au cours de notre étude, seuls des sporanges parasitant des cellules végétatives ont été reportés. Il a été montré que la composition macromoléculaire des cellules végétatives différait de celle des akinètes (Sutherland*, et al.*, 1979). De plus, les capacités métaboliques de ces deux types cellulaires diffèrent. De précédentes études ont pu

mettre en évidence que les akinètes matures présentent un métabolisme réduit, en raison notamment d'une diminution importante de la respiration et, par conséquent, une faible consommation ou production d'énergie (Fay, 1969, Chauvat*, et al.*, 1982). Par conséquent, nous émettons l'hypothèse suivante : plus que la « quantité », la qualité (composition macromoléculaire, métabolisme) des hôtes pourrait impacter le développement et notamment la fécondité de leurs chytrides parasites.

Chapitre 5

Discussion générale & Perspectives

Il est désormais établi que les Chytridiomycota (chytrides) constituent les principaux pathogènes fongiques du phytoplancton, cependant, la majeure partie des travaux menés sur les interactions phytoplancton-chytride s'est attachée à étudier le parasitisme fongique de la composante eucaryote du phytoplancton (Kagami & Urabe, 2002, De Bruin*, et al.*, 2004, Ibelings*, et al.*, 2011), délaissant l'étude des interactions entre les pathogènes et les cyanobactéries, alors même que cette composante constitue la principale biomasse phytoplanctonique en période estivale dans les écosystèmes lacustres. Au vu des enjeux économiques, sociétaux et écologiques que représentent les blooms cyanobactériens, il nous a semblé essentiel de mieux comprendre l'impact des parasites fongiques, facteur biotique encore largement méconnu, pouvant influencer la dynamique cyanobactérienne. C'est dans ce cadre que s'inscrivent les travaux conduits au cours de ma thèse, centrés sur les associations hôte-parasite impliquant les cyanobactéries et les chytrides. Ces travaux ont été menés sur le lac d'Aydat qui constitue un site particulièrement intéressant pour l'étude des dynamiques cyanobactériennes, puisque des *blooms* de cyanobactéries filamenteuses appartenant à l'espèce *Anabaena macrospora* se développent de manière récurrente en période estivale. Cette espèce cyanobactérienne, de par sa morphologie filamenteuse, est connue pour être récalcitrante à la prédation zooplanctonique. L'influence des autres facteurs biotiques, tel que le parasitisme fongique pourrait, par conséquent, avoir des implications écologiques non négligeables, en rapport avec le contrôle de la dynamique de blooms cyanobactériens.

Une meilleure connaissance des systèmes hôte-parasite mettant en jeu les cyanobactéries et les chytrides semble donc nécessaire pour mieux comprendre les voies de régulation biotique des blooms cyanobactériens. Plus globalement, au-delà de cette vision « modélistique », l'étude du système cyanobactérie/chytride offrira une vision plus précise du fonctionnement des réseaux trophiques mis en place lors de phase de proliférations cyanobactériennes monospécifiques dans les écosystèmes aquatiques. Ainsi les travaux conduits au cours de cette thèse nous ont permis i) d'évaluer les impacts des chytrides sur la dynamique de la population cyanobactérienne ii) de caractériser les facteurs contrôlant la dynamique et iii) la fécondité des parasites fongiques ; et iv) d'identifier les conséquences (contrôle des populations phytoplanctoniques, rôle des zoospores dans le transfert de matière aux niveaux trophiques supérieurs…) de ce parasitisme dans le fonctionnement du réseau trophique aquatique. L'ensemble de ces travaux ont nécessité la mise au point de protocoles méthodologiques permettant une analyse microscopique fine des systèmes phytoplancton-chytrides et du cycle de développement des chytrides.

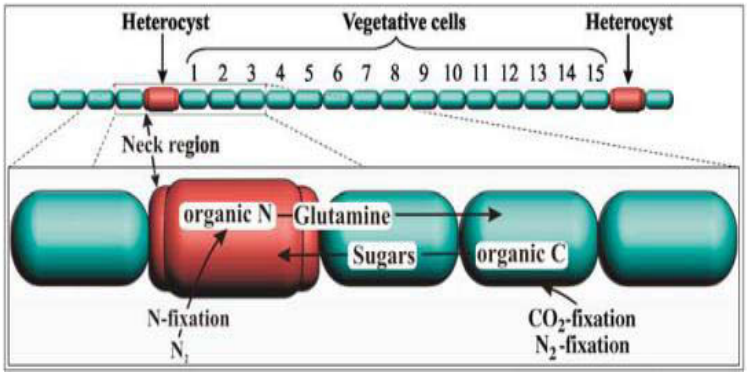

Figure 1 : Modèle de fixation et de transport du CO_2 et du N_2 dans un filament d'*Anabaena oscillarioides* présentant deux hétérocystes. Le nombre indique les cellules végétatives à différentes distances de l'hétérocyste. D'après Popa *et al.,* 2007

I. Impact des chytrides sur la dynamique cyanobactérienne : deux espèces fongiques aux impacts distincts

En parasitant les cellules végétatives grâce à son système tubulaire rhizoïdal, *Rhizosiphon crassum* impacte directement la biomasse active des blooms cyanobactériens lors de leur développement (Article 3). Cependant, les faibles prévalences d'infection de cette espèce fongique rapportées en 2010 et 2011 ne permettent pas de désigner cette espèce comme le principal facteur mais plutôt comme un des facteurs biotiques responsables du déclin du bloom d'*Anabaena macrospora*. Les cellules parasitées par *R. crassum* sont, en effet, littéralement détruites et ne participent donc plus au développement de la biomasse cyanobactérienne. En plus de cet impact direct du parasitisme conduisant à la mort de plusieurs cellules au sein d'un filament, *R. crassum* pourrait également avoir un effet sur la physiologie de l'ensemble du filament. En effet, un filament cyanobactérien est le siège de nombreux échanges de métabolites carbonés et azotés (Popa*, et al.*, 2007) (Fig. 1). En tuant plusieurs cellules végétatives, *R. crassum* peut limiter les échanges de matière au sein du filament et dans le cas des cyanobactéries hétérocystées, comme c'est le cas d'*Anabaena macrospora*, diminuer l'apport d'azote nécessaire à la croissance des cellules végétatives et l'apport de molécules carbonées vers les hétérocystes, essentielles pour un fonctionnement optimal. Ainsi, l'impact du chytride pourrait s'étendre à l'ensemble du filament parasité. Cette hypothèse pourrait être vérifiée au travers d'expérimentations combinant l'utilisation d'isotopes stables (^{13}C et ^{15}N) et de microscopie ionique à haute résolution de type NanoSIMS. Ainsi, la comparaison des flux d'azote et de carbone circulant au sein de filaments parasités et de filaments non parasités, nous permettra de caractériser et de quantifier précisément l'impact de l'espèce *R. crassum* sur la physiologie de son hôte et ainsi de parfaire nos connaissances sur l'impact du parasitisme fongique sur la dynamique cyanobactérienne. Cette perspective nécessite la mise en place et le maintien de cultures pures de notre système hôte-parasite d'intérêt. Ceci pourrait paraitre chose aisée mais la mise en place et surtout le maintien d'une culture d'un couple hôte parasite est complexe et actuellement aucune culture chytride-cyanobactérie n'est disponible. En effet, afin de maintenir un parasite en culture il est essentiel de connaitre la durée de son cycle de vie, au risque de perdre l'ensemble de la co-culture. Même si notre travail mené en 2010 a permis d'acquérir ce paramètre (Article 3), et d'estimer la durée du cycle de développement de *R. crassum* à environ 3jours, l'isolement de nombreux filaments parasités réalisés lors de *blooms* et placés dans diverses conditions expérimentales, n'a pas conduit au maintien de notre couple

hôte-parasite en conditions de laboratoire. D'autres investigations seraient alors nécessaires car la mise en culture de chytrides parasites représente un enjeu scientifique majeur pour la prise en compte du parasitisme dans le fonctionnement des réseaux trophiques microbiens.

Le développement d'un système tubulaire rhizoïdal au travers de plusieurs cellules du filament diminuerait l'adhérence cellule à cellule de la colonie (pour une revue bibliographique sur la compartimentalisation et l'adhérence cellule-cellule dans les filaments cyanobactériens, voir Flores & Herrero, (2010), conduisant à une fragmentation mécanique des filaments d'*Anabaena macrospora*, phénomène que nous avons pu observer et mettre en évidence lors de notre première étude *in situ* (Article 3) et précédemment suggéré par Sigee *et al.,* (2007) sur *Anabaena flos-aquae*. Chan et collaborateurs (2004) ont rapporté que dans le cas de cyanobactéries hétérocystées, la fragmentation des filaments pouvait physiologiquement affecter la population cyanobactérienne en supprimant la capacité de fixation de l'azote atmosphérique, et ainsi accélérer le déclin des blooms cyanobactériens. Cette fragmentation conduit à la réduction de la taille des filaments hôtes pouvant alors faciliter leur ingestion. Par ailleurs, la réduction de la taille des filaments pourrait faciliter leur dispersion dans la colonne d'eau, dispersant ainsi le couple hôte-parasite de façon homogène dans l'ensemble de la masse d'eau, tant en milieu pélagique central que littoral (Article 4).

Parallèlement à l'infection par *R crassum*, nous avons mis en évidence un second chytride, *R akinetum*. Son action parasitaire se distingue en tous points de celle de *R. crassum*. En parasitant les akinètes matures, *R. akinetum* ne participe pas directement au déclin des *blooms* cyanobactériens. En revanche, son impact sur l'inoculum cyanobactérien, i.e. la densité des akinètes dans les sédiments, n'est pas à négliger notamment lorsque la prévalence d'infection est importante, comme nous avons pu le rapporter lors de l'année 2011 (jusqu'à 36% des akinètes parasités) (Articles 4 et 5). En effet, il a été montré que la densité de l'inoculum influençait la date de mise en place du *bloom* cyanobactérien (Tsujimura & Okubo, 2003, Tsujimura, 2004). Dans le cas des cyanobactéries formant des *blooms* en fin d'été voire en période automnale, comme *A. macrospora*, plus la mise en place du *bloom* sera tardive, plus la période de conditions abiotiques favorables, sera courte. Ceci pourrait alors réduire la durée et l'intensité du *bloom* cyanobactérien. Par ailleurs, la structure génétique des populations de cyanobactéries planctoniques est liée à la structure génétique des populations benthiques résistant dans les sédiments durant l'hiver (Misson, Sabard *et al.,* en préparation). En parasitant les seules cellules de résistance de la population cyanobactérienne d'*Anabaena macrospora*, *R. akinetum* pourrait fortement impacter la structure génétique de la population

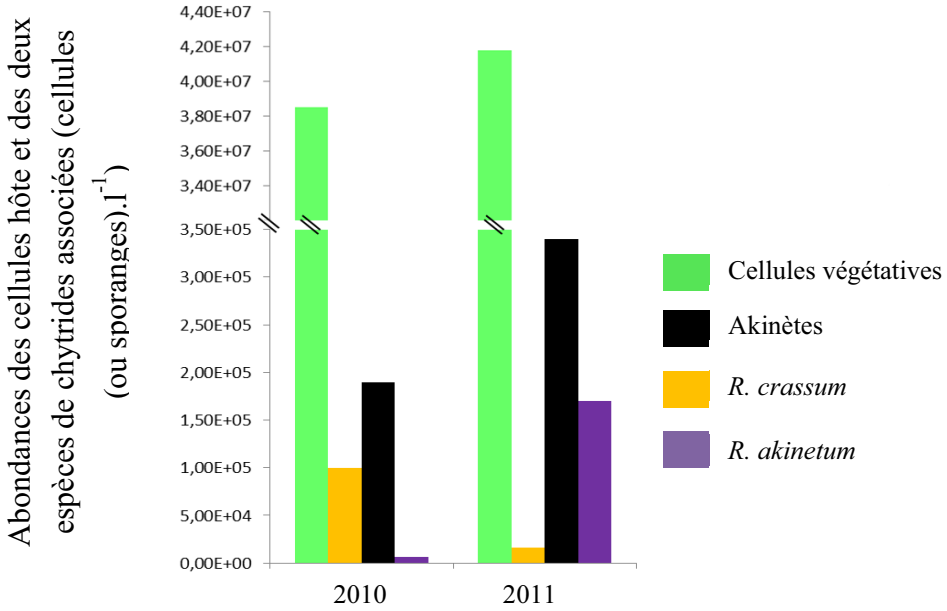

Figure 2 : Comparaison interannuelle des maxima d'abondance des hôtes (cellules végétatives (vert) et akinètes (noir)) et des parasites fongiques associés (*Rhizosiphon crassum* (jaune) et *Rhizosiphon akinetum* (violet)).

cyanobactérienne. *R. akinetum* n'a pas d'impact sur la dynamique cyanobactérienne de l'année n. En revanche, ce chytride pourrait profondément modifier la structure génétique de la population de l'année n+1 et ainsi avoir un rôle non négligeable dans l'évolution de la diversité génétique de la population hôte. Cette hypothèse ouvre des perspectives intéressantes sur l'étude comparative de la structure des communautés benthiques et planctoniques de cyanobactéries, et des facteurs contrôlant les liens existant entre ces deux phases du cycle de vie des cyanobactéries. Une étude visant à comparer la génétique des akinètes planctoniques sédimentant l'année n à des akinètes benthiques recrutés l'année n+1 lors d'épidémie de *R. akinetum* permettrait de tester notre hypothèse sur l'influence de ce parasite dans le contrôle de la structure génétique des cellules hôtes.

II. Facteurs de contrôle de la dynamique hôte-parasite dans les écosystèmes aquatiques

Les deux espèces fongiques étudiées au cours de ces travaux de thèse se distinguent par leur i) type cellulaire hôte, ii) leur développement et iii) leur fécondité. De plus, l'étude de deux années successives a révélé un changement de dominance de ces deux espèces fongiques (Fig. 2). Le parasitisme peut être influencé par de nombreux facteurs, tant biotiques (densité, composition moléculaire, physiologie, diversité génétique de l'hôte) qu'abiotiques (température).

1) L'hôte : facteur déterminant de la dynamique du parasite

a) Relation cyanobactérie-chytrides : sommes-nous face à une relation-densité dépendante ?

Le développement des parasites obligatoires telles que les deux espèces de chytrides rencontrées au cours de cette thèse est dépendant de leur hôte. En effet, l'hôte constitue la seule ressource énergétique indispensable à l'accomplissement de leur cycle de vie. De ce fait, la présence de l'hôte dans le milieu est une condition *sine qua non* à l'infection parasitaire. De nombreux auteurs ont pu mettre en évidence que les systèmes hôte-parasite mettant en jeu le phytoplancton et les chytrides, requéraient une densité minimale d'hôtes dans le milieu pour la mise en place de l'infection fongique de type épidémique (Paterson, 1960, Sen, 1988, Kudoh & Takahashi, 1992, Holfeld, 1998). Au cours du suivi temporel réalisé lors de l'efflorescence cyanobactérienne de 2010 (Article 3 ; Gerphagnon *et al.,* 2013), nous avons

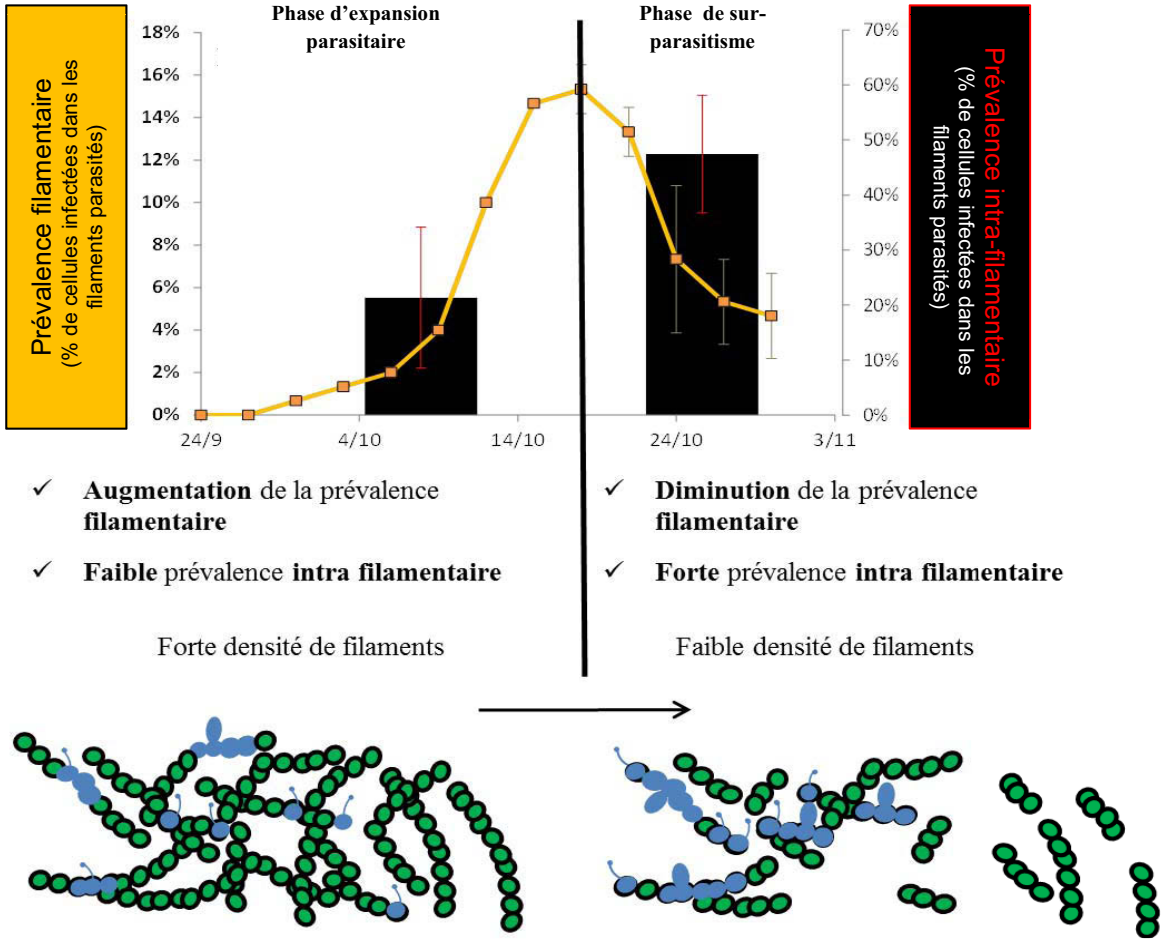

Figure 3 : Illustration des deux phases de l'infection fongique observées au cours du suivi temporel de l'année 2010. Les cellules végétatives d'*Anabaena macrospora* sont représentées en vert, les chytrides *R. crassum*, en bleu.

rapporté que les infections parasitaires dues aux chytrides *R.crassum* et *R. akinetum* se déclenchaient à partir de densités différentes, respectivement de 1.5×10^4 cellules végétatives.ml^{-1} et 1.8×10^3 akinètes.ml^{-1}. Ibelings *et al.* (2004) ont émis l'hypothèse suivante : plus l'espèce hôte présente un biovolume important, plus le seuil minimal de densité requis pour induire l'infection est faible. Cette hypothèse est vérifiée à l'échelle de notre système puisque les akinètes, qui présentent des biovolumes 10 fois supérieurs en moyenne à celui des cellules végétatives, présentent une densité minimale d'hôtes requise 13 fois inférieure à celle de ces dernières. Cependant, au cours de notre étude (Article 5 ; Gerphagnon *et al.,* (2013)) nous avons clairement mis en évidence que la taille de l'hôte ne pouvait pas être l'unique facteur conduisant à l'infection fongique, puisqu'aucune différence significative n'avait alors pu être mise en évidence entre les biovolumes des akinètes infectés et ceux des akinètes non infectés. En revanche, des différences de concentration en divers composés tels que les carbohydrates, existent entre les cellules végétatives et les akinètes. Les akinètes présentent en effet des concentrations plus importantes de réserves glucidiques (Sutherland*, et al.*, 1979). En milieu marin, Muehlstein *et al.,* (1988) désignent les carbohydrates comme des composés impliqués dans l'attraction chemotactique d'un chytride vers son hôte. La concentration plus importante en réserves glucidiques des akinètes comparativement aux cellules végétatives pourrait conduire à un chemotactisme plus important de *R. akinetum* vers son hôte, que ne le font les cellules végétatives pour *R. crassum*. Ceci pourrait alors expliquer que l'infection des akinètes par *R. akinetum* requiert un seuil minimal d'hôtes plus faible.

L'étude des dynamiques fines du système *Anabaena macrospora-Rhizosiphon sp.* a mis en exergue le lien étroit existant entre la dynamique des parasites et celle de leurs hôtes. En effet, au travers de l'étude temporelle menée en 2010 (Article 3) il est clairement apparu que l'infection parasitaire présentait deux phases distinctes (Fig. 3) : une première phase d'expansion parasitaire mise en place lors de forte densité d'hôte, suivie d'une seconde phase de « sur-parasitisme » apparaissant lors du déclin du bloom cyanobactérien. Lors de la phase d'expansion parasitaire une augmentation significative de la prévalence filamentaire (PrF) atteignant la valeur maximale de 17% des filaments, ainsi qu'une faible prévalence cellulaire au sein de ces filaments (PrCF) ont été rapportées. En étudiant le cycle de vie de ce chytride nous avons pu relier cette expansion parasitaire à la forte production puis la libération des zoospores dans le milieu (Article 3), et ainsi montrer qu'en condition naturelle l'ensemble de la population de *R. crassum* présentait une synchronisation de son cycle de vie. Suite à cette

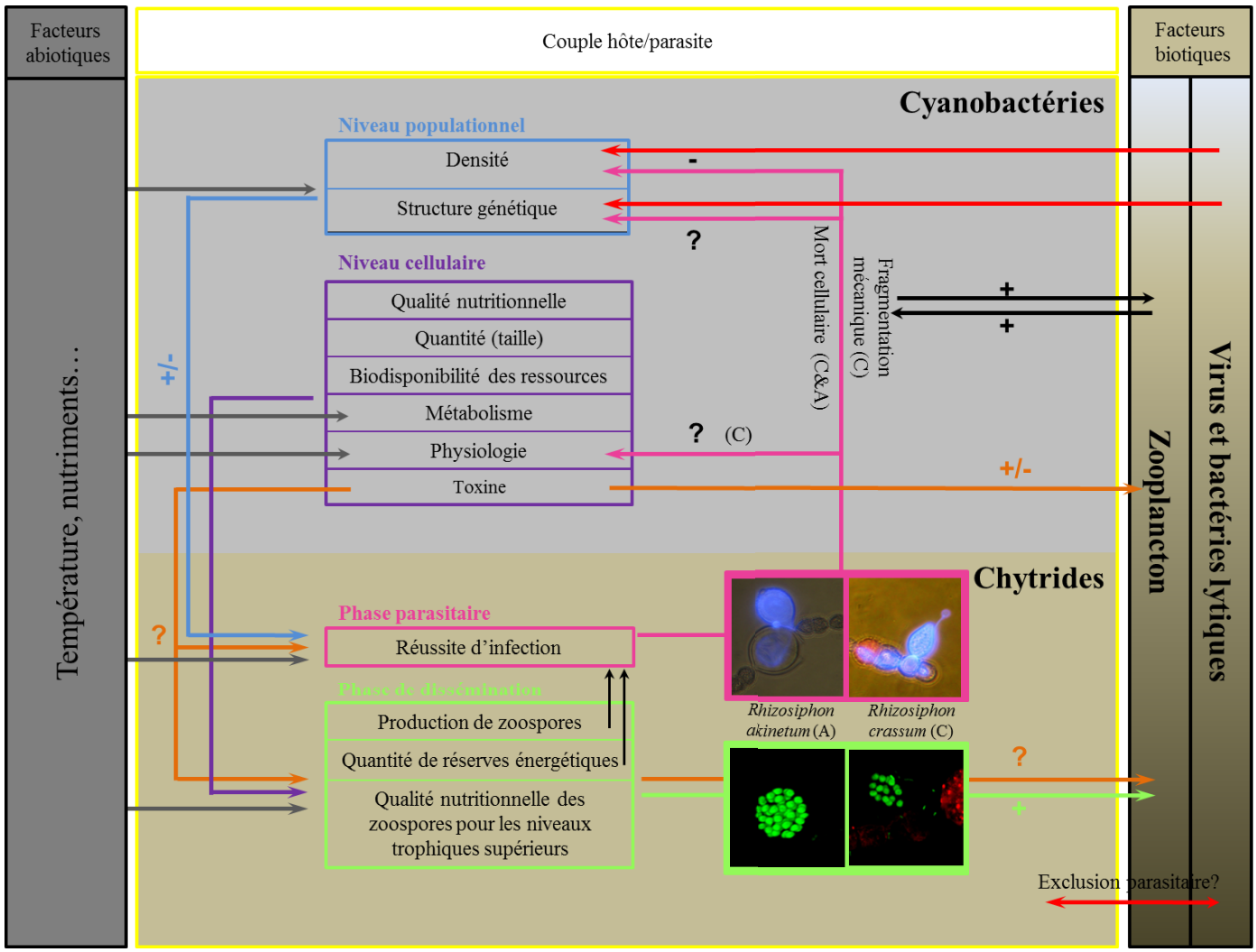

Figure 4: Facteurs biotiques et abiotiques impactant le couple cyanobactérie-chytride

Facteurs de la population (**bleu**) et de la cellule (**violet**) hôte impactant les chytrides.

Facteurs de la phase parasitaire (**rose**) impactant l'hôte.

Paramètres de la phase de dissémination (**vert**) pouvant impacter le zooplancton.

Potentiel impact des cyanotoxines (**orange**)

Interactions du couple hôte-parasite avec les virus et les bactéries lytiques (**rouge**).

A: *Rhizosiphon akinetum*, C: *Rhizosiphon crassum*

phase d'expansion, la phase de « sur-parasitisme » est caractérisée par la diminution progressive de la prévalence filamentaire, et une forte augmentation de la PrCF (Fig. 3). Des études précédentes ont montré que le chemotactisme était l'un des mécanismes expliquant l'attraction des chytrides vers leur hôte (Powell, 1994, Moss*, et al.*, 2008). Concernant les chytrides parasites du phytoplancton, les excrétions phytoplanctoniques seraient les principaux métabolites participant à ce chemotactisme (Bruning, 1991, Powell, 1994, Moss*, et al.*, 2008, Sonstebo & Rohrlack, 2011). De ce fait, de fortes densités d'hôtes seraient un facteur important favorisant le taux de rencontre, mais également favorisant l'attraction des zoospores. En effet, Kudoh & Takahashi (1992) ont pu montrer qu'une zoospore ne pouvait explorer qu'un faible volume d'eau (40µl) lors de son temps de vie. Ainsi, lorsque les densités d'hôtes dans le milieu sont faibles, comme c'est le cas lors de la phase de « sur-parasitisme», les nouvelles zoospores libérées dans le milieu pourraient être plus attirées par les excrétions de cellules situées au sein du filament déjà infecté que par celles de filaments non infectés, trop éloignés pour un chemotactisme efficace.

Par ailleurs, l'étude spatiotemporelle menée en 2011 (Article 4) nous a permis d'établir qu'il n'existait pas de refuge pour les cyanobactéries face aux chytrides, les parasites fongiques ayant été retrouvés en milieu pélagique au centre du lac mais également au niveau du littoral sans aucune différence significative.

Bien que la densité de l'hôte semble jouer un rôle primordial dans la dynamique hôte parasite, elle ne constitue pas l'unique paramètre de contrôle du parasitisme fongique (Fig. 4). En effet, en 2011, la population cyanobactérienne présentait une densité de cellules végétatives significativement plus importante que l'année précédente (Fig. 2), pourtant leur parasite *R. crassum*, exhibait une abondance et une prévalence d'infection significativement plus faibles qu'en 2010. De plus, la comparaison des deux proliférations cyanobactériennes étudiées, révèle une inversion de dominance de nos deux espèces fongiques (Fig. 2). Aux vues de nos résultats et de la littérature, il apparait clairement que la densité de l'hôte n'est pas l'unique facteur intervenant dans l'intensité des infections parasitaires. En effet, d'autres paramètres comme la température (Bruning, 1991) ou la structure génétique de la population de l'hôte peuvent également intervenir.

b) Importance de la structure génétique de la population hôte dans la relation parasitaire

Des études ont montré que les chytrides, et plus précisément leur virulence, était intiment liées à la génétique de leurs hôtes, qu'ils soient eucaryotes (De Bruin*, et al.*, 2004) ou procaryotes (Sonstebo & Rohrlack, 2011). En d'autres termes, ces auteurs ont pu mettre en évidence que les chytrides étaient plus dépendants du génotype que de l'espèce d'hôte. En présentant une telle spécificité, les systèmes hôte-parasite impliquant le phytoplancton et les chytrides s'inscrivent parfaitement comme modèles de la théorie de la reine rouge « Red Queen Hypothesis » (De Bruin*, et al.*, 2008). Développée par Leigh Van Valen (1935-2010) cette théorie métaphorique symbolisant la course aux armements des systèmes biologiques, est basée sur la co-évolution des hôtes et de leurs parasites : les hôtes maintiennent une importante activité de remaniement génétique pour résister aux parasites qui, à leur tour, mettent en place des mécanismes moléculaires pour franchir ces barrières de résistance. Ainsi, de nombreuses études ont pu mettre en évidence, tant en milieu marin que lacustre, une importante diversité génétique des populations cyanobactériennes lors d'efflorescence algale (Wilson*, et al.*, 2005, Briand*, et al.*, 2008, Fewer*, et al.*, 2009, Ye*, et al.*, 2009). Soumise aux contraintes environnementales, une sélection de génotypes adaptés aux conditions abiotiques (et biotiques) présentes, s'opère. Ainsi, non seulement des remodelages de structure génétique peuvent se produire au cours de la prolifération cyanobactérienne (Briand*, et al.*, 2008), notamment pendant la phase de croissance du bloom (Pobel*, et al.*, 2012), mais des modifications structurales peuvent également se produire d'une année à l'autre (Tanabe & Watanabe, 2011). Les processus liés à ces modifications de structure génotypique ne sont pas clairement établis mais les chytrides pourraient jouer un rôle important (Fig. 4). Avec cette vision de coévolution des systèmes hôtes-parasites, les dynamiques de nos couples *Anabaena macrospora-Rhizosiphon sp*. au cours de ces 5 dernières années sur le lac d'Aydat sont très informatives. L'étude menée par Rasconi en 2007 (Rasconi*, et al.*, 2012) n'a rapporté l'existence que d'une seule espèce fongique : *R. crassum*. Cependant des valeurs extrêmement élevées de prévalence filamentaire (max. 98% des filaments étaient infectés) ont été rapportées cette année-là, suggérant une sensibilité très importante de la population cyanobactérienne face à cette espèce fongique. La non-détection de l'espèce *R. akinetum* en 2007 pourrait provenir i) de sa très faible prévalence d'infection ou ii) d'un biais d'échantillonnage. En effet nous avons pu montrer lors de notre première étude (**Article 3**) toute l'importance d'un échantillonnage haute fréquence en rapport avec le temps de

génération des systèmes hôte-parasite. Or, en 2007 la fréquence d'échantillonnage n'était que bi-mensuelle et la fenêtre d'infection des akinètes par *R. akinetum* aurait pu être manquée. En 2010, des prévalences filamentaires beaucoup plus faibles ont été rapportées (max. 17% des filaments étaient infectés), et 4% des akinètes étaient atteints par l'espèce *R. akinetum*. Enfin, en 2011 seul 2,5% des filaments étaient parasités par *R. crassum.* En revanche *R. akinetum* exhibait une prévalence d'infection maximale de 36% des akinètes matures.

En 5 ans, la population cyanobactérienne semble être devenue de plus en plus résistante à *R. crassum* mais de plus en plus sensible à *R. akinetum*. Cette situation ne semble pas correspondre à une exclusion parasitaire puisque les deux espèces fongiques possèdent des types cellulaires hôtes distincts. De plus, des observations, bien que très largement minoritaires, de ces deux espèces fongiques parasitant un seul et même filament ont été faites, prouvant leur possible coexistence. En revanche, l'évolution de notre système pourrait résulter d'une coévolution de nos couples hôte-parasite tendant vers une résistance de la population cyanobactérienne à *R.crassum*, et d'une plus forte sensibilité à *R. akinetum*. Pour donner quelques éléments de réponse à cette hypothèse, il serait intéressant d'étudier la structure génétique de l'ensemble de la population cyanobactérienne. Des approches « *single-filament* » pour l'étude de filaments parasités par l'une ou l'autre de nos espèces fongiques permettraient de « screener » l'ensemble des génotypes sensibles ou résistants à nos espèces. Ainsi, l'évolution temporelle des proportions de génotypes sensibles et résistants aux chytrides permettrait d'appréhender *i)* une partie des mécanismes de défense mis en place par l'hôte et *ii)* l'impact de ces espèces fongiques sur la structuration génotypique de nos populations hôtes. Par ailleurs, ces expérimentations permettraient de répondre à des questions complémentaires; elles permettraient notamment de vérifier que le concept formulé par Thingstad et Lignell (1997) pour les systèmes bactéries-virus, « killing the winner», est applicable aux systèmes phytoplancton-chytride. Van Donk (1989) a pu montrer que les chytrides favorisaient les successions phytoplanctoniques en parasitant la population dominante, ce qui laisse supposer que ce concept est bien applicable à notre système à l'échelle populationnelle. Cependant, qu'en est-il au niveau génotypique? Ce concept est-il applicable à cette échelle ? Le génotype dominant est-il différemment affecté par l'infection fongique que les autres génotypes ? Si oui, est-il le plus sensible, ou au contraire le plus résistant aux chytrides ? Autant de questions en perspective qui permettraient de mieux comprendre les interactions entre le phytoplancton et leurs champignons parasites.

2) La température : un facteur abiotique influençant la dynamique hôte-parasite

De nombreux auteurs ont pu mettre en évidence que la température du milieu conditionne la physiologie des communautés aquatiques, les relations trophiques, et peut également fortement influencer la virulence des infections parasitaires (Gillooly*, et al.*, 2002, Hall*, et al.*, 2006, Brooks & Hoberg, 2007, Masclaux*, et al.*, 2009). Les études menées sur les couples diatomées-chytrides, tant en conditions naturelles qu'en conditions expérimentales, ont révélé l'importance de ce facteur abiotique sur les dynamiques hôte-parasite.

Si de nombreuses études ont rapporté la préférence des cyanobactéries pour des températures élevées (Sommer*, et al.*, 1986, Robarts & Zohary, 1987, Paerl & Huisman, 2008, Whitton, 2012, Zhang*, et al.*, 2012), peu d'études se sont attachées à étudier les preferendum thermiques de leurs parasites fongiques (Paterson, 1960, Barr & Hickman, 1967). Cependant, l'étude menée par Paterson (1960) sur la dynamique du système *Anabaena planctonica-Rhizosiphon Anabaenae* rapporte des prévalences d'infection importantes lorsque la température lacustre est élevée (22°C à 26°C). De plus, Barr & Hickman (1967), ont montré en conditions expérimentales que *Rhizophidium sphaerocarpum,* chytride parasite de la cyanobactérie *Spirogyra sp.,* présentait un optimum de croissance à une température également très élevée (30°C). Ces quelques travaux corroborent les résultats que nous avons pu obtenir au cours de nos deux études *in situ*. En effet, les températures relevées dans l'épilimnion au cours de la prolifération cyanobactérienne de l'année 2011 présentaient des valeurs de 1 à 3°C plus élevées que l'année précédente. Cette élévation de température s'est accompagnée d'une plus forte biomasse de la population cyanobactérienne et d'une augmentation globale de l'abondance parasitaire (Article 3 et 4). De tels résultats soutiennent les prévisions de nombreux auteurs, qui, dans un contexte de changement global, se sont interrogés sur l'impact potentiel de l'augmentation de la température des eaux sur les infections parasitaires (Fig. 4). La plupart de ces auteurs prévoit une augmentation significative du parasitisme dans les années futures, parallèlement à la hausse des températures (Harvell*, et al.*, 2002, Lafferty & Kuris, 2005, Studer*, et al.*, 2010, Macnab & Barber, 2012). Cependant, les travaux menés par Ibelings *et al*. (2011) ne semblent pas vérifier cette hypothèse générale connue sous l'expression « *warmer hence sicker world* ». En étudiant sur une période de 30 ans le couple *Asterionella formosa-Rhizophidium planktonicum*, ces auteurs ont conclu que des températures plus élevées conduisaient à une réduction de la prévalence parasitaire sans pour autant bénéficier à la population hôte. Cependant, ces travaux ont été conduits sur des systèmes hôtes-parasites impliquant des

diatomées, dont l'optimum thermique est relativement bas. Les cyanobactéries présentent des préférendum thermiques plus élevés que ceux des diatomées, leurs parasites pourraient par conséquent être plus adaptés à des températures élevées et ainsi proliférer plus abondamment avec une augmentation de la température. Cependant, si à l'échelle globale, le parasitisme fongique des cyanobactéries semble être favorisé par une élévation de la température, l'ensemble de nos travaux rapporte des réponses différentes à l'échelle inter-spécifique. Ainsi, alors que la prévalence d'infection due à *R. crassum* ne semble pas être affectée par un abaissement de la température (Article 3), celle de *R. akinetum*, en revanche, marque une diminution parallèlement à des chutes brutales de température et semble favorisée par des températures moyennes plus élevées (Article 3 et 4). Ces différences d'impact de ce paramètre abiotique en fonction de l'espèce fongique peuvent correspondre à des stratégies d'infection différentes de la part de ces deux espèces. Des études plus fines visant à préciser les réponses de ces deux systèmes hôte-parasite face aux variations des températures permettraient de mieux appréhender l'impact des chytrides sur les populations cyanobactériennes dans le cadre du réchauffement global.

La dynamique des populations hôte-parasite, comme toute dynamique de population, est conditionnée par la fécondité des individus. Aussi, nous nous sommes interrogés sur les facteurs conditionnant la fécondité de nos parasites. Pour cela, une méthode permettant d'étudier les relations existantes entre l'hôte et le parasite, et notamment l'impact de l'hôte sur la fécondité fongique, a été développée (Chapitre 4) et offre des éléments de réponse intéressants

III. Facteurs influençant la fécondité des chytrides

1) Influences de la quantité et de la qualité de l'hôte

De l'énergie contenue dans la cellule hôte dépendra la taille finale du sporange et par conséquent le taux de production de zoospores (fécondité). Ainsi dans l'Article 5 (Gerphagnon *et al.* 2013) et les résultats complémentaires associés, nous avons pu mettre en évidence l'étroite relation existante entre la taille de l'hôte parasité (reflétant la quantité d'énergie disponible pour les chytrides), et la capacité de production des deux espèces de chytrides étudiées : *Rhizosiphon akinetum* et *R.crassum*. Plus la quantité d'hôte parasité par un chytride est importante, plus la taille finale du sporange est importante, et par

conséquent, plus le nombre de zoospores produites est important. Cependant, il semble que la taille de l'hôte ne soit pas l'unique facteur déterminant la capacité de production des zoospores. En effet, outre la quantité, il semblerait que la « qualité » de l'hôte joue un rôle important (Fig. 5). Les résultats issus de différentes études (Chapitre 4) et de la littérature (Bruning, 1991), ont révélé des capacités de production de zoospores de 2,5 à 5,5 fois plus élevées pour les espèces de chytrides parasitant des diatomées que pour les chytrides infectant la cyanobactérie *Anabaena macrospora*. Contrairement aux diatomées, les cyanobactéries sont considérées comme un groupe phytoplanctonique présentant une qualité nutritionnelle médiocre, en raison notamment de l'absence de stérols et d'acides gras polyinsaturés à longue chaine (Muller-Navarra*, et al.*, 2000, Von Elert*, et al.*, 2003). De plus, Lord and Roberts (1986) ont rapporté que la production de zoospores de *Lagenidium giganteum*, un oomycète parasite de moustique, était dépendante de la qualité de la nourriture. L'ensemble de ces données nous amène à suggérer que la qualité nutritionnelle de l'hôte pourrait fortement influencer la fécondité des chytrides (Fig. 4).

2) <u>La disponibilité des ressources nutritionnelles du parasite</u>

A l'échelle interspécifique, la comparaison de la fécondité de nos deux espèces de chytrides a révélé des disparités (Fig. 5). *R. crassum* présente une capacité de production de zoospores 1,5 fois plus élevée que celle de *R. akinetum*. La composition lipidique de leur hôte (respectivement cellule végétative et akinète) ne présente pourtant que de très faibles différences (Yamamoto, 1972). Ce facteur ne semble donc pas pouvoir être le seul facteur explicatif des différences observées.

Si les cellules végétatives ne diffèrent pas des akinètes d'un point vue lipidique, elles présentent cependant une composition macromoléculaire et un métabolisme différents de celui des akinètes. En effet lors de leur différenciation et de leur maturation les akinètes accumulent des réserves énergétiques telles que la cyanophycine (réserve azotée) et le glycogène (réserve glucidique). Les akinètes matures présentent ainsi 8 fois plus de cyanophycine et 16 fois plus de glycogène que les cellules végétatives (Sutherland*, et al.*, 1979). Le glycogène est un carbohydrate indispensable aux chytrides. En effet, il constitue l'une des principales énergies de réserve glucidique des zoospores lorsque ces dernières sont émises dans le milieu.

Cependant, bien qu'ayant accès à une quantité plus importante de réserves énergétiques, *R. akinetum* présente une plus faible capacité de production de zoospores. Néanmoins, aux vues de la complexité de cette molécule, il semble peu probable que le glycogène puisse être directement absorbé et assimilé par le chytride sans être tout d'abord catabolisé en une molécule plus simple. La question de la biodisponibilité de la ressource semble donc se poser (Fig 4). La comparaison du développement et de la production de zoospores par *R. akinetum* cultivé sur différents milieux riches en molécules simples tel que le glucose, ou complexes comme le glycogène, permettrait sans doute d'apporter quelques éléments de réponse. Néanmoins, aux vues de nos résultats, la quantité de glycogène contenue dans l'hôte ne semble pas constituer un avantage pour le parasite (Chapitre 4). Cependant, pourrait-elle influencer la concentration en glycogène des zoospores ? Il serait intéressant d'étudier s'il existe une relation entre la quantité de réserve glucidique de l'hôte et celle contenue dans les zoospores. En effet, ceci pourrait avoir un impact important dans le cycle de vie des chytrides puisque le temps de vie des zoospores est fonction de la quantité initiale en réserves énergétiques (Holfeld, 2000). De ce fait, si les zoospores contiennent plus de réserves énergétiques, leur temps d'exploration et de ce fait la probabilité de rencontre avec l'hôte seraient plus importants, améliorant ainsi le succès d'infection du parasite.

3) *Influence du métabolisme de l'hôte*

Les études menées par Bruning (1991) ont révélé que le métabolisme de l'hôte pouvait impacter le temps de développement du sporange et le nombre de zoospores produites. Lors de leur différenciation les akinètes sont métaboliquement très actifs mais cette activité diminue au cours de leur maturation jusqu'à devenir quasiment nulle (Fay, 1969, Rao*, et al.*, 1984). *R. akinetum* ne parasite que les akinètes ayant accompli leur phase de maturation. Ces akinètes matures présentent une faible activité métabolique. En revanche, *R. crassum* quand à lui parasite des cellules végétatives métaboliquement actives et présente un taux de fécondité plus important (Article 3 et Chapitre 4). Ainsi, la différence notable d'activité métabolique rapportée entre les akinètes et les cellules végétatives pourrait en partie expliquer la plus grande fécondité de *R. crassum* (Fig. 4). Afin de tester cette hypothèse, le passage en conditions expérimentales semble indispensable. Ainsi, il serait intéressant de comparer la capacité de production de zoospores de l'espèce *R. crassum* lorsque ce dernier parasite des cultures d'hôtes en phase stationnaire, en phase exponentielle de croissance, ou en phase de déclin, reflétant différents degrés d'activité métabolique des hôtes.

Stratège-r	Stratège-K
Capacité reproductive élevée	Capacité reproductive faible
Parasite des stades "jeunes" de leur hôte	Parasite les derniers stades de cycle de vie de leur hôte
Tendance à l'opportunisme	Très spécialisés
Vivent dans des environments présentant des grandes disponibilité de ressources	Vivent dans des environments présentant de faible disponibilité de ressources
Petite taille	Grande taille
Bonne tolérance aux perturbations environmentales	Faible tolérance aux perturbations environmentales
Développment rapide	Développement lent

Tableau 1 : Comparaison relative des deux espèces fongiques parasites de la cyanobactérie *Anabaena macrospora* dans un modèle r et K. ***Rhizosiphon crassum*** est représenté par la couleur beige, ***R. akinetum*** par la couleur violette. Lorsqu'aucune couleur ne figure, ceci signifie que les données ne sont pas connues. (Les caractéristiques des deux stratégies sont issues d'une étude menée par Barbosa, 1977)

IV. Synthèse et réflexion autour des stratégies écologiques adoptées par les deux espèces fongiques étudiées

Si *R. crassum*, grâce à son rhizoïde tubulaire, a la capacité d'infecter les cellules végétatives et les akinètes de la cyanobactérie *Anabaena macrospora* (bien que ce dernier cas ne soit que très peu rencontré), *R. akinetum* quant à lui possède une niche cellulaire plus spécifique, puisque réduite aux akinètes, stade ultime du cycle de vie annuel des Nostocales (Article 3). De plus, *R. crassum* exhibe une taille plus petite que *R. akinetum* (Chapitre 4). En revanche ce chytride est plus fécond puisqu'il possède une capacité de production de zoospores 1,5 fois supérieure à celle de *R. akinetum* (Article 5). L'ensemble de ces résultats mettent en évidence deux stratégies écologiques différentes de ces deux espèces fongiques. Il a été suggéré précédemment, que de la stratégie écologique adoptée par un parasite, dépendait sa capacité de contrôle exercée sur son hôte (Pianka, 1970, Force, 1972, Ehler & Miller, 1978, Wiedenmann & Smith, 1997). Développée en 1967 par les deux écologues Robert MacArthur et E. O. Wilson (MacArthur & Wilson, 1967), le modèle r/K, a donné naissance aux concepts d'espèces à stratégie r et d'espèces à stratégie K, dont les principales caractéristiques sont recensées dans le tableau 1. Ces auteurs ont pu mettre en évidence que la stratégie-r était plus avantageuse pour les parasites. En effet, en présentant des cycles de vie courts et une fécondité importante, le parasite peut facilement outrepasser le temps de vie de son hôte et de ce fait exercer un contrôle plus important que les espèces présentant une stratégie K. Par ailleurs, la coexistence de parasites stratèges r et K infectant une même espèce hôte a préalablement été mis en évidence chez des macroorganismes (Barbosa, 1977). Les deux espèces fongiques arborent des stratégies écologiques différentes, et même s'il est délicat de les catégoriser en stratèges r ou K avec les éléments dont nous disposons, il semble néanmoins que *R. akinetum* est visiblement plus proche d'un stratège K que *R crassum* (tableau 1).

Les stratégies écologiques de ces deux champignons diffèrent profondément mais sont-elles le reflet d'une réelle spéciation ? Tout en affirmant être face à deux stratégies écologiques foncièrement différentes, nous sommes cependant en droit de nous interroger sur la distance phylogénétique séparant les deux chytrides étudiés. En effet, la discrimination de ces deux champignons n'est basée que sur des traits morphologiques préalablement décrits par Canter (1954). Il serait ainsi nécessaire de séquencer et d'analyser les génomes de ces

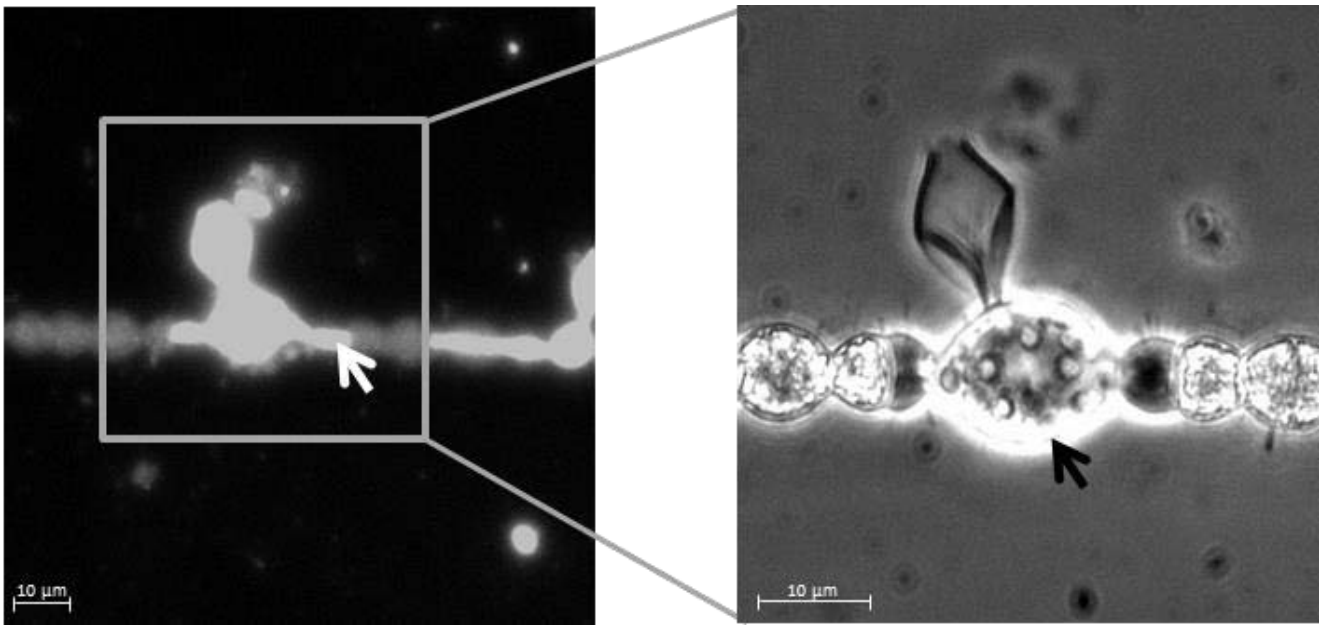

Figure 6 : *Rhizosiphon crassum* parasitant un akinète (flèche noire) et développant un système rhizoïdal tubulaire (flèche blanche) au travers de plusieurs cellules d'un filament cyanobactérien d'*Anabaena macrospora*.

deux entités biologiques afin de déterminer si nous sommes effectivement face à deux espèces ou deux sous-espèces distinctes. Dans ces cas, l'acquisition ou la perte de gènes au cours de l'évolution auraient permis une spéciation des deux parasites ayant conséquemment entrainé une spécialisation sur deux types de cellules et des modes d'infection différents (i.e. avec ou sans rhizoïde). Les deux champignons pourraient également avoir divergé de leur ancêtre commun bien en amont et de ce fait ne pas appartenir au même genre. Il y aurait alors eu une convergence de deux entités très éloignées vers une seule et même espèce hôte se « partageant » et s'adaptant aux différentes ressources disponibles (i.e. différents types cellulaires) dans les filaments cyanobactériens. Enfin, nous pourrions également être face à une seule et même espèce présentant différents phénotypes induits par le type cellulaire parasité. Ce dernier cas de figure est cependant improbable puisque nous avons pu observer lors de nos études des individus fongiques parasitant des akinètes et présentant toutes les caractéristiques morphologiques de *R. crassum* (forme ovoïde du sporange, système rhizoïdal tubulaire) (Fig. 6).

V. Rôle des chytrides dans les réseaux trophiques aquatiques : la réflexion continue

En parasitant la biomasse active des cyanobactéries, nous avons pu montrer que les chytrides réduisaient directement jusqu'à 6% de la biomasse cyanobactérienne active (Article 3). A cet impact direct, il faut rajouter l'impact physiologique potentiel sur l'ensemble du filament, ainsi que la fragmentation mécanique induite par les chytrides (Article 3). La taille des filaments cyanobactériens est une des adaptations permettant aux cyanobactéries de limiter l'impact du broutage sur leur population. Cependant, certaines espèces zooplanctoniques sont capables de brouter efficacement des filaments de petite taille (Bouvy, et al., 2001, Oberhaus, et al., 2007). En réduisant la taille des filaments, les chytrides favoriseraient le broutage des cyanobactéries par le zooplancton et en ce sens promouvraient le déclin des blooms cyanobactériens.

Outre l'impact de la phase parasitaire, la phase de dissémination des chytrides, les zoospores, pourraient faire tomber le dogme de l'impasse trophique cyanobactérienne, créant un véritable lien entre espèces phytoplanctoniques « *inedible* » et le zooplancton. Les travaux de thèse menés sur la phase de dissémination des chytrides, nous ont permis de mettre en évidence des

différences significatives de production de zoospores reliées au groupe phytoplanctonique parasité (Article 5). La discussion émanant de ces résultats, appuyée notamment sur les travaux de Lord & Roberts (Lord & Roberts, 1986), a conduit à émettre l'hypothèse de l'influence de la qualité nutritionnelle de l'hôte sur la production fongique. Il serait très intéressant d'explorer cette piste à l'avenir. Une expérimentation mettant en jeu une même espèce fongique parasitant deux espèces hôtes distinctes appartenant au même taxon nous permettrait de comparer l'importance de la composition biochimique de l'hôte sur la production fongique.

Au-delà de l'impact de cette qualité nutritionnelle sur la production de zoospores, les travaux menés par Kagami *et al.,* (2007) suggèrent que la composition lipidique (acide gras et stérols) du parasite est en lien étroit avec celle de l'hôte. Par ailleurs, Kagami *et al,.* (2004) et Kagami *et al.,* (2011) ont pu montrer que les zoospores de *Rhizophydium planktonicum* pouvaient être broutées et assimilées par les cladocères et les copépodes. De plus, les eucaryotes flagellés sont capables de produire de novo des stérols, lipides non produit par les cyanobactéries mais indispensables au développement des arthropodes (Von Elert, *et al.,* 2003). Les zoospores, au même titre que les flagellés, pourraient contribuer au « trophic upgrading » des cyanobactéries (Bec, *et al.,* 2006). Il serait ainsi très intéressant de mener une étude expérimentale sur la composition lipidique des zoospores de diverses espèces fongiques parasitant des groupes phytoplanctoniques contrastés d'un point de vue biochimique. Par exemple, la comparaison de la composition lipidique des zoospores de *Rhizosiphon crassum*, parasite de la cyanobactérie *Anabaena macrospora*, et celle de *Rhizophydium planctonicum*, parasite de la diatomée *Asterionella formosa*. Ceci nous permettrait de quantifier, et de comparer, l'importance de l'apport nutritionnel des zoospores pour les niveaux trophiques supérieurs lors de proliférations d'espèces phytoplanctoniques « *inedible* » eucaryotes et procaryotes.

Outre leur faible qualité nutritionnelle, certaines cyanobactéries peuvent être potentiellement toxiques. Récemment, Rohrlack et collaborateurs (2013) ont étudié le potentiel impact des métabolites secondaires produits par les cyanobactéries du genre *Planktothrix* sur l'intensité d'infection fongique. Ces auteurs ont ainsi pu montrer que les souches productrices, notamment de microcystine, bien que parasitées de manière non négligeable (jusqu'à 50% de prévalence d'infection) étaient significativement moins infectées que leurs mutantes non productrices de toxine, ce qui suggère que les métabolites secondaires pourraient jouer un rôle de défense face au parasitisme fongique. Cependant, ces auteurs ne se sont pas intéressés au

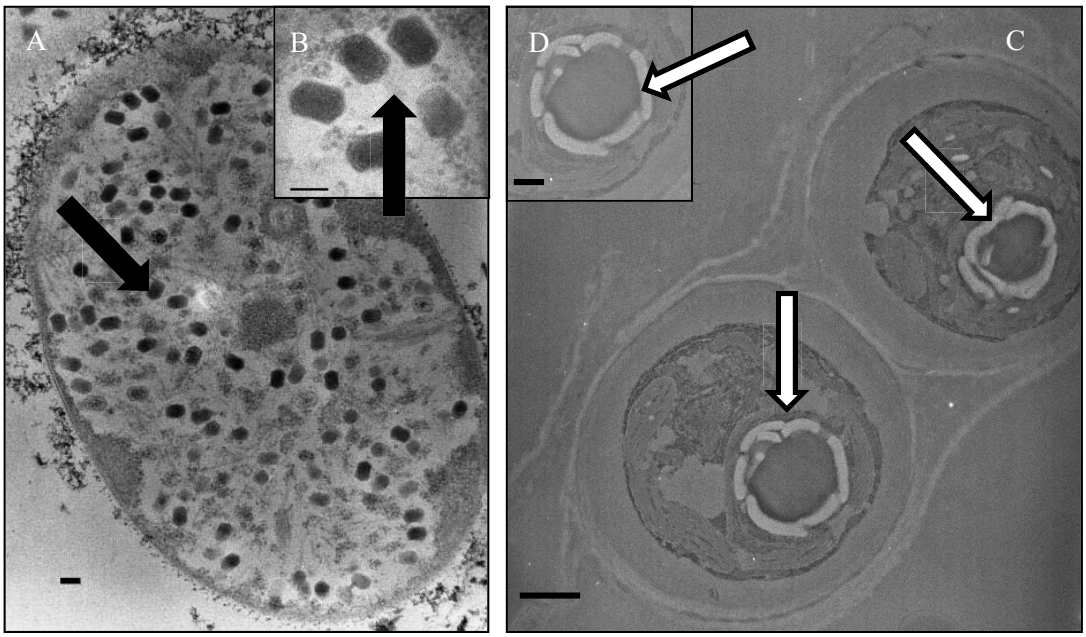

Figure 7: Photographies issues d'observation au MET d'un akinète infecté par des virus à queue (siphoviridae) (flèche noire) (A, B) et de deux cellules végétatives parasitées par le système rhizoïdal tubulaire de *Rhizosiphon crassum* (flèche blanche)(C, D). Echelle : 100nm (B, D) ; 1µm (A, C). Photo : Jonathan Colombet

devenir de cette toxine dans les parasites fongiques ayant réussi à « outrepasser » cette défense. Assimilent-ils cette toxine comme une ressource carbonée ou l'accumulent-ils? Si les chytrides peuvent jouer un rôle important en tant que lien entre matière phytoplanctonique « *inedible* » et zooplancton, pourraient-ils également permettre la « détoxification » des cyanobactéries pour le zooplancton (Bec *et al.,* 2006) ou au contraire transférer cette toxine vers les niveaux trophiques supérieurs (Sopanen*, et al.*, 2009)? La réponse à l'ensemble de ces questions permettrait d'améliorer nos connaissances sur les flux de matière et d'énergie de la dernière boucle trophique décrite : la « mycoloop » (Kagami, *et al.,* 2007).

Par ailleurs, lors de notre échantillonnage temporel à haute fréquence (2010), nous avons pu effectuer des observations au microscope électronique à transmission. Des rhizoïdes fongiques ainsi que des virus, vraisemblablement des siphoviridae, ont ainsi pu être mis en évidence au sein de cellules végétatives et d'akinètes (Fig. 7). Cependant aucune cellule cyanobactérienne infectée par les deux entités biologiques n'a pu être observée. Existe-t-il une exclusion parasitaire ? Quelle est l'importance relative de ces deux types de parasites dans le contrôle des blooms cyanobactériens? Leur impact étant toujours considéré séparément, aucune information n'émerge concernant leur synergie. Ainsi, une étude spatio-temporelle visant à étudier parallèlement les trois boucles trophiques (virale, microbienne et fongique) lors de prolifération cyanobactériennes nous permettrait de connaitre s'il existe d'une part (i) un découplage séquentiel de ces dernières dans les écosystèmes lacustres, et d'autre part (ii) une ségrégation spatiale entre ces trois boucles au sein de la colonne d'eau.

Enfin, une étude comparative des apports nutritifs de la mycoloop et de la boucle microbienne vers les niveaux trophiques supérieurs permettrait de clairement quantifier l'importance de ces boucles dans le transfert de matière et d'énergie lors de proliférations d'espèces phytoplanctoniques « *inedible* » considérés comme des impasses trophiques pour les niveaux trophiques supérieurs, et plus globalement d'obtenir une meilleure estimation de la productivité des écosystèmes lacustres.

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Zhao, S., Wei-bin, P., and Chao, M. (2012) Stimulation and inhibition effects of algae-lytic products from *Bacillus cereus* strain L7 on *Anabaena flos-aquae*. *Journal Applied Phycology* **24**: 1015-1021.

Annexes

Curriculum Vitae

Scientific publications

Gerphagnon M, Latour D, Colombet J, Sime-Ngando T. (2013) A double staining method using SYTOX-green and Calcofluor White for studying fungal parasites of phytoplankton. *Applied and Environmental Microbiology* 79:3943-3951.

Gerphagnon M, Colombet J, Latour D, Sime-Ngando T. (2013) Fungal parasitism: life cycle, dynamics and impact on cyanobacterial blooms *PLoS ONE 8:e60894.*

Sime-Ngando T, Rasconi S and **Gerphagnon M.** (2013) Diagnosis of parasitic fungi in the plankton: technique for identifying and counting infective chytrids using epifluorescence microscopy, p. 169-174. In V. K. Gupta, M. G. Tuohy, M. Ayyachamy, K. M. Turner, and A. O'Donovan (ed.), Laboratory Protocols in Fungal Biology. Springer New York.

Monchy S, Jobard M, Sanciu G, Rasconi S, **Gerphagnon M**, Chabé M, Cian A, Meloni D, Niquil N, Christaki U, Viscogliosi E and Sime-Ngando T. (2011): Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing, *Environmental microbiology,* **13**, 1443-1453

Gerphagnon M, Sime-Ngando T (2011): Cover photograph illustrating the topic of the Hydrobiologia Special Issue, T. Sime-Ngando & N. Niquil (Guest Editors) / Disregarded Microbial Diversity and Ecological Potentials in Aquatic Systems. Multiple infections of an unknown colonial Chlorophyceae by zoosporic fungi (Chytrids) sampled in the surface waters of the eutrophic Lake Aydat, Massif Central, France. Apparently young sporangia penetrate into the mucilage and reach the cell wall but without rhizoidal system inside the host cells, highlighting the thin boundary between parasitism and saprophytism. *Hydrobiologia*, Special Issue, Volume **659**, Issue 1

Manuscripts submitted or in preparation

Gerphagnon M, Macarthur D.J, Gachon C, Van Ogtrop F, Latour D, Lilje O, Gleason F, Sime-Ngando T. The biological factors affecting the dynamics of cyanobacterial blooms. Submitted.

Gerphagnon M, Monchy S, Sime-Ngando T. Comparasion Seasonal eukaryotic community structure in a eutrophic lake: from microscopy to tag pyrosequensing In preparation.

Gerphagnon M, Sime-Ngando T, Latour D. Spatiotemporal distribution of aquatic fungal parasitism: the case of chytrid-cyanobacterium pairings. In preparation.

Gerphagnon M, Batisson I, Latour D. Cyanobacterial blooms: friendship or hostility between a bacterial community not previously exposed to a bloom event and *Microcystis aeruginosa*? In preparation.

Communications

2012 August	**14th International Symposium on Microbial Ecology,** Copenhagen (Denmark), **M. Gerphagnon**, J. Colombet, D. Latour and T. Sime-Ngando "Freshwater cyanobacterial bloom is connected to the grazing food chain via fungal parasitism" (Poster)
2012 May	**Internal seminar of phD students,** Clermont Ferrand (France), **M. Gerphagnon,**J. Colombet, D. Latour and T. Sime-Ngando "Fungal parasites: their contributions to the decline of cyanobacteria in freshwater lakes" (Poster)
2012 April	**9ème Rencontre des microbiologistes,** Clermont Ferrand (France), **M. Gerphagnon**, J. Colombet, D. Latour and T. Sime-Ngando "Parasitism: a missing factor involved in the decline of cyanobacterial blooms" (Oral communication)
2011 June	**7th SEFS (Symposium European of Freshwater Sciences)**, Girona (Spain), **M. Gerphagnon**, J. Colombet, D. Latour and T. Sime-Ngando "Chytrid parasitism: a missing factor involved in the decline of cyanobacterial blooms" (Oral communication)
2011 January	**8th Congress of GRISCYA (GRoupe d'Intérêt Scientifique sur les CYAnobactéries),** Clermont Ferrand (France), **M. Gerphagnon**, J. Colombet, D. Latour and T. Sime-Ngando "Temporal evolution of fungi parasitism associated to a cyanobacterial bloom" (Oral communication)
2010 January	**7th Congress of GRISCYA (GRoupe d'Intérêt Scientifique sur les CYAnobactéries),** Bourg en Bresse (France), **M. Gerphagnon**, I.Batisson and D. Latour. "Impact of microcystin LR on bacterial community: an experimental approach" (Oral communication)

Teaching and Supervision

2012-2009 Teaching **at Université Blaise Pascal (Clermont Ferrand, France) (192h)**

- Theorical Ecology (Licence 3)
- Aquatic Ecology (Licence 1 and 2)
- Animal Biology (Licence1 and 2)
- Parasitology (Licence 2)

2010 Supervisor of Valérie Péres (Master I) Obj: "Study of phytoplankton and fungi parasitism in a eutrophic lake". Duration: 3 months

Implication in Scientist Projects

-French ANR Programme Blanc « **ROME** : **R**are and **O**verlooked **M**icrobial **E**ukaryotes in aquatic ecosystems »

-French ANR Programme Blanc « **DREP**: **D**iversité et **R**ôles des **E**umycètes dans le **P**élagos »

-Sakura Project: Collaboration between France and Japan